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Molecular mechanisms of intestinal epithelial cell proliferation and apoptosis - the role of pro-inflammatory cytokines

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- the role of pro-inflammatory cytokines

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SUMMARY

Normal intestinal epithelial cell turnover is characterized by a sequence of proliferation-differentiation-apoptosis. Immature stem cells, located in the basal crypt compartment, possess a high mitotic activity. Their daughter cells gradually differentiate into mature enterocytes during their migration along the crypt-villus axis. Once reaching the villus tip, these post-mitotic mature intestinal epithelial cells ultimately die via apoptosis and are extruded into the intestinal lumen. The underlying regulatory and molecular mechanisms controlling this highly dynamic sequence are still largely unknown. There is increasing evidence that cytokines and certain growth factors play an important role in regulating homeostatic intestinal epithelial cell turnover. Alterations in the production of these regulatory factors, as seen in chronic idiopathic inflammatory bowel disorders (IBD), are thought to contribute to the markedly disturbed intestinal epithelial cell turnover that characterize these disorders. Morphological analysis of actively inflamed IBD tissue revealed an exaggerated rate of premature cell death of intestinal epithelial cells via apoptosis, leading to villus atrophy, as well as a compensatory crypt hyperplasia. The major aim of this research was to uncover the mechanisms by which pro-inflammatory cytokines result in pathological enterocyte turnover, with special emphasis on intestinal epithelial cell proliferation and apoptosis. Four different, complementary *in vitro* models (IEC-6, HIEC, Caco-2 cell lines and *ex vivo* colon explant cultures) were used to analyze the effect of pro-inflammatory cytokines and the underlying mechanisms of theses changes. In a first step, we identified interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα) as potent modulators of immature intestinal crypt cell growth. In the IEC-6 cell model, TNFα induced proliferation was mediated by the MAP kinase signaling cascade, similar to that observed in response to epidermal growth factor (EGF). On the other hand, IFNγ arrested immature crypt cells in the quiescent G_0/G_1-phase of their cell cycle, completely inhibiting enterocyte cell growth. Costimulation with IFNγ and TNFα revealed a clear signaling hierarchy between these two cytokines, with the anti-proliferative effect of IFNγ dominating. Depending on cytokine concentration and TNF receptor stimulated, TNFα played a dual cytotrophic and cytotoxic role for intestinal epithelial cells, as demonstrated by growth promotion or induction of apoptosis. Further

analysis of the signaling mechanisms underlying TNFα's effects revealed that ICE-caspases were critical in triggering apoptosis, whereas caspase-3 was not involved. In marked contrast, butyrate-induced Caco-2 cell apoptosis was strongly dependent on this particular protease, indicating distinct signaling pathways in response to specific pro-apoptotic stimuli. Complete inhibition of the caspase-cascade resulted in necrotic cell death after stimulation with TNFα. Based on these findings, we speculate that the caspase-cascade may play an important protective role against necrotic cell death. In addition, IFNγ and TNFα are also involved in the regulation via the FAS pathway, as demonstrated by our studies using HIEC and *ex vivo* human colonic explant cultures. Both cytokines markedly enhanced the susceptibility of enterocytes to undergo apoptosis upon stimulation of FAS. An important underlying mechanism was the upregulation of enterocyte FAS-expression. The effects of these pro-inflammatory cytokines were not limited to the alteration of intestinal epithelial cell turnover, but also extended to the cross-talk between immune and intestinal epithelial cells. In this study, we analyzed the regulation of enterocytic MHC class II expression by pro-inflammatory cytokines, as an example of enterocyte-immune cell interactions. IFNγ and TNFα were found to be potent upregulators of immature crypt cell class II expression, potentially leading to an altered intestinal antigen handling and pathological immune responses. Taken together, our data provide clear evidence that pro-inflammatory cytokines such as IFNγ and TNFα, act directly on intestinal epithelial cells. Both are implicated in the regulation of intestinal epithelial cell turnover and simultaneously serve as important mediators of the intercellular cross-talk that occurs in the intestinal mucosa. Based on our findings, it is evident that alterations in the production and secretion of these pluripotent cytokines locally in the mucosa potentially results in deleterious changes in the closely regulated intestinal epithelial cell turnover and consequently in intestinal function.

RESUME

L'épithélium intestinal constitue un des tissus au renouvellement le plus rapide du corps humain. Il est caractérisé par la séquence prolifération–différenciation-apoptose. Dans la base des cryptes, se situent les cellules souches immatures ayant une grande capacité mitogénique. Leur cellules filles se différencient graduellement pendant leur migration tout au long de l'axe crypte-villosité. Arrivées au pic des villosités, ces cellules postmitotiques et matures meurent par apoptose. Les mécanismes moléculaires, ainsi que le système de contrôle de ce renouvellement très dynamique demeurent encore largement inconnus. Des travaux récents ont proposé que certaines cytokines ou facteurs de croissance pourraient jouer un rôle très important dans la régulation du renouvellement des cellules épithéliales intestinales. Ceci a été démontré pour le facteur de croissance de transformation (TGF) α et β. Des altérations de la production et de la sécrétion de différentes cytokines ou facteurs de croissance pourraient donc être la cause des perturbations majeures de la muqueuse intestinale observées chez des patients atteints d'une maladie inflammatoire chronique de l'intestin, telle que la maladie de Crohn ou la colite ulcéreuse. En effet, ces maladies immunitaires de l'intestin sont caractérisées par une prépondérance de cytokines pro-inflammatoires sécrétées par les cellules immuno-compétentes recrutées dans la muqueuse intestinale inflammée.

L'hypothèse principale de ce projet de recherche est la suivante: Les cytokines pro- inflammatoires interféron gamma (IFNγ) et tumor necrosis factor alpha (TNFα) sont des facteurs très importants dans la pathogénèse des maladies inflammatoires de l'intestin. Ces cytokines interviennent directement sur le renouvellement des cellules intestinales épithéliales en modifiant et régularisant la capacité mitogénique et l'apoptose des entérocytes. Ceci entraine une destruction nette de l'épithélium intestinal comme on le voie chez les patients atteints d'une maladie inflammatoire chronique de l'intestin. Dans les lésions inflammatoires chez ces patients, on détecte des entérocytes

apoptotiques tout au long de l'axe crypte-villosité d'une façon exagérée et prématurée. Normalement, les cellules apoptotiques sont limitées à la surface des villosités de l'intestin grêle et au sommet des cryptes du colon. Quelques rares cellules apoptotiques peuvent être détectées à la base des cryptes dans la zone de prolifération. L'apoptose précoce des entérocytes entraîne une perte majeure de cellules matures et une diminution considérable de la capacité d'absorption qui se traduit cliniquement par une diarrhée et une malabsorption. Nous avons plus particulièrement cherché à démontrer les mécanismes moléculaires de ces événements. Nous avons choisi quatre modèles complémentaires comme approche expérimentale: Les lignes cellulaires IEC-6 (cellules immatures non-transformées de rat), HIEC (cellules humaines immatures non-transformées), Caco-2 (cellules humaines cancéreuses, partiellement différenciées) et des biopsies de colon humain cultivées ex vivo.

Dans une première étape, nous avons pu déterminer que les deux facteurs interféron gamma (IFNγ) et tumor necrosis factor alpha (TNFα) agissent comme modulateurs de la croissance des cellules épithéliales intestinales immatures. L'IFNγ est un puissant inhibiteur de la prolifération de ces cellules qui agit en les bloquant dans la phase G_0/G_1, la phase de repos du cycle cellulaire. Par contre, le TNFα s'est révélé être un stimulateur de la prolifération des cellules IEC-6. L'analyse du rôle des deux récepteurs au TNFα, p55 et p75, a permis d'identifier que l'effet promitogénique de cette cytokine est exclusivement médié par le récepteur p55. La stimulation de ce récepteur entraîne une activation de la cascade de MAP kinases, identique à celle du facteur de croissance épidermal (EGF). Le rôle clé des kinases Erk1/2 dans la signalisation de TNFα a été vérifié par western blot. L'inhibition de la phosphorylation de ces enzymes par l'inhibiteur spécifique, PD98059, bloque complètement la réponse proliférative au TNFα.

Le TNFα peut avoir un double rôle sur le renouvellement de l'épithélium intestinal. La même cytokine peut être cytotrophique ou cytotoxique. En plus de la

stimulation de la prolifération des cellules IEC-6, le TNFα peut également induire la mort cellulaire par apoptose. Pour déclencher l'apoptose des cellules intestinales immatures par activation isolée du récepteur p55, des doses de TNFα de 1000 ng/ml ou plus sont nécessaires. Par contre, une concentration 100 fois moindre de cytokine est suffitsante pour déclencher la même réponse si les deux récepteurs sont stimulés en même temps. Nos observations supportent largement le concept que le récepteur p75 au TNF joue un rôle de potentiateur dans l'induction de l'apoptose. En analysant les voies de signalisation, nous avons observé qu'une activation des caspases de type ICE est indispensable pour générer une réponse apoptotique. L'inhibition des ICE-caspases abolit complètement l'apoptose induite par le TNFα. Par contre, l'inhibition spécifique de la caspase-3 est sans effet dans notre modèle d'apoptose. Des analyses par western blot ont confirmé que le TNFα ne stimule pas cette caspase. Contrairement au TNFα, l'apoptose des cellules cancéreuses, induite par le butyrate, est dépendante de la caspase-3, mais pas des caspases du type ICE. Ceci montre que différents stimuli proapoptotiques peuvent utiliser des voies de signalisation distinctes pour induire l'apoptose d'une cellule épithéliale intestinale.

De manière surprenante, l'inhibition complète de la cascade de caspases avec le peptide zVAD-fmk, qui, à faible dose, a des propriétés anti-apoptotiques, change la réponse apoptotique au TNFα en une forme nécrotique de mort cellulaire. Ces expériences indiquent, que la cascade de caspases peut aussi avoir un rôle protecteur contre la nécrose. Donc, dépendamment des facteurs additionnels, le TNFα peut induire la mort cellulaire des cellules épithéliales intestinales par apoptose ou nécrose. Ceci indique la présence de deux voies de signalisation différentes empruntées par le TNFα.

Un autre mécanisme très efficace pour déclencher l'apoptose d'une cellule cible est la voie de signalisation de FAS-FAS ligand. En utilisant le modèle HIEC, nous avons observé que ces cellules sont résistantes à l'apoptose médiée par ce récepteur. Par contre, la présence de costimulateurs, tel que les cytokines pro-inflammatoires IFNγ ou TNFα, rend ces cellules épithéliales intestinales immatures sensibles à l'apoptose après

stimulation du récepteur FAS. L'analyse des mécanismes moléculaires de ce phénomène révèle que l'IFNγ et le TNFα sont des stimulateurs très puissants de l'expression de FAS. La combinaison des deux cytokines a un effet synergique. Ces observations in vitro ont pu être confirmées avec des biopsies du colon normal, cultivées ex vivo. L'effet de sensibilisation de l' IFNγ sur l'apoptose induite par FAS a été documenté par TUNEL-assay in situ. A faibles doses, FAS ne déclenche pratiquement pas d'apoptose dans ces explants, mais la costimulation avec IFNγ induit l'apoptose des cellules matures et immatures de l'épithélium intestinal en détruisant la structure intestinale. Ces résultats indiquent que non seulement ces facteurs pro-inflammatoires peuvent induire la mort cellulaire par apoptose, mais en même temps elles rendent les cellules épithéliales intestinales beaucoup plus sensibles à l'apoptose déclenchée par d'autres récepteurs, tel que FAS.

Les cytokines pro-inflammatoires sont aussi impliquées dans la modulation de l'interaction entre les cellules intestinales épithéliales et les cellules du système immunitaire. Dans ce travail, nous avons étudié l'effet de ces cytokines sur le récepteur CHM de class II des entérocytes. IFNγ ainsi que TNFα sont des stimulateurs de l'expression des molécules classe II par les cellules cryptes immatures. Le TNFα tout seul est sans effet, mais augmente l'action d' IFNγ d'une façon synergique. La néo-expression de molécules de classse II sur les cellules cryptes ou une expression augmentée sur les entérocytes peut altérer la présentation des antigènes par les cellules épithéliales intestinales. Ceci pourrait entraîner des changements pathologiques de la réponse immunitaire.

En conclusion, nos résultats montrent que les cytokines pro-inflammatoires TNFα et IFNγ sont des modulateurs très puissant du renouvellement entérocytaire. Les deux facteurs interagissent directement sur les cellules épithéliales intestinales, mais en même temps, ces facteurs sont également impliqués dans le cross-talk entre les cellules du système immunitaire et l'épithélium intestinal. Basé sur nos résultats, il est facilement compréhensible, que des changements de l'équilibre physiologique des

cytokines peuvent causer des perturbations majeures du renouvellement entérocytaire, tel qu'observé chez des patients atteints d'une maladie inflammatoire de l'intestin. Nos résultats montrent que l'induction d'apoptose est un effet très important du TNFα. Le progrès dans la compréhension des mécanismes moléculaires de cet effet, pourrait potentiellement aider a créer des nouveaux médicaments avec des propriétés anti-apoptotiques.

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List of Abbreviations

DFF	DNA-fragmentation factor
EGF	epidermal growth factor
ERK1/2	extracellular signal-regulated kinase
FADD	FAS associated death domain
I-kappa B	inhibitor of kappa B
ICAM-1	intercellular adhesion molecul-1
ICE	interleukin converting enzyme
IFN	interferon
IL	interleukin
KGF	keratinocyte growth factor
MAP	mitogen activated protein
MEK	mitogen-activated protein (MAP) kinase/Erk kinase
MHC	major histocompatibility complex
NF-kappa B	nuclear factor-kappa B
p55 TNF-R	p55 TNF-receptor
p75 TNF-R	p75 TNF-receptor
PARP	poly-(ADP-ribosyl) polymerase
PS	phosphatidyl serine
SAP	stress activated protein
STAT	signal transducers and activator of transcription
TGF	transforming growth factor
TNF	tumor necrosis factor
TRADD	TNF-receptor associated death domain
TRAF	TNF-receptor associated factor

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I. INTRODUCTION

The role of apoptosis in the regulation of intestinal epithelial cell turnover

The intestinal epithelium is a highly dynamic cell population characterized by a complete turnover every 48 to 96 hours (Potten et al., 1992). Normal intestinal function and morphology depends upon a delicate balance in cell types and numbers from immature proliferating crypt cells to highly differentiated enterocytes which undergo apoptosis at the end of their life span. The proliferating immature stem cells are located in the basal crypt compartment. Their daughter cells gradually differentiate into mature cell types (enterocytes, goblet cells or enteroendocrine cells) during their migration along the crypt-villus axis. Once reaching the villus tip in the small bowel or crypt summit in the colon, these highly differentiated postmitotic cells die via apoptosis and are extruded into the lumen (Iwanaga et al. 1993, Hall et al. 1994, Potten et al. 1992, Strater et al. 1995). Apoptosis, the process associated with physiological cell death, is thus the endstage of migration and differentiation of intestinal epithelial cells along the crypt-villus axis. This programmed cell death is the counterpart to crypt cell proliferation. The homeostatic balance between apoptosis and proliferation is essential to normal gut morphology and function (Ruemmele and Seidman, 1998). Exaggerated or accelerated cell dying via apoptosis results in paucity of mature enterocytes leading to villus atrophy, whereas uncontrolled crypt cell proliferation leads to pathological crypt hyperplasia. An efficient and very closely regulated control system is thus a prerequisite to guarantee a functional steady state.

One might question the teleological relevance of such an active rate of proliferation and subsequent differentiation on one side, as well as apoptosis rate on the other, necessitating a complicated regulatory system. One hypothesis suggests that rapid proliferation/differentiation and loss of terminally differentiated cells via apoptosis prevents accumulation of mutated cells, potentially leading to malignant transformation (Podolsky 1993). In fact, the intestinal epithelium is at high risk of tumoral

transformation due to its exposure to various mutagens present in the intestinal lumen. The relatively high incidence of colon cancers supports the relevance of this issue and underlines the necessity of suitable protective mechanisms for this particular organ. However, there is a clear discrepancy between the occurrence of neoplasia in the small bowel and colon, indicating that despite high turnover rates and identical genotypic background, additional factors, such as the presence of intraluminal bacteria, might contribute to the pathogenesis of intestinal tumors.

The closely regulated normal sequence of proliferation-differentiation-apoptosis along the crypt-villus axis can be disturbed at one or more levels, as observed in various pathological conditions (Moss et al. 1996, Leichtner et al. 1996 a,b, McCullen et al. 1997). For example, a significant dysregulation of intestinal epithelial cell turnover is documented in patients with inflammatory bowel disease (IBD), a chronic recurrent inflammatory state of the intestinal tract of yet unknown origin. The two distinct subtypes, Crohn's disease (CD) and ulcerative colitis (UC), are both characterized by an inflammatory destructive process of the intestinal mucosa, resulting in erosions and ulcerations (Crohn 1967). Whereas in UC the inflammatory process is continuous and restricted to the colon, in CD any portion of the entire intestinal tract, from the mouth to the anus, can be affected with discrete regions of inflammation and intact intervening mucosa (Leichtner et al. 1996 a,b). Morphological analysis revealed a dramatically increased number of dying intestinal epithelial cells at the site of acute inflammation, characterized by an inflammatory infiltrate in form of activated lymphocytes, neutrophils and monocytes. On the other hand, crypt elongation is also frequently encountered in IBD tissue, indicating a compensatory hyperproliferative state. Further analysis of the underlying mechanisms revealed that intestinal epithelial cells die preferentially via apoptosis in immune-mediated bowel disorders. Under normal conditions, apoptotic cells are only found at the villus tip/crypt summit, whereas in the basal crypt compartment, only slight apoptosis occurs, if any at all (Strater et al. 1995, Moss et al. 1996, McCullen et al. 1997). However, in IBD patients, this pattern is completely disturbed, with a great number of apoptotic cells observed along the entire crypt-villus axis (Strater et al. 1997, Iwamoto et al. 1997). This abnormal increase in premature death of differentiating enterocytes via apoptosis, combined with the

excessive proliferation of stem cells, leads to a functional loss of normal absorptive capacity along with an inappropriate secretion of intestinal fluid and electrolytes, typical of immature crypt epithelial cells (Weaver 1996). For the patient, this is manifested clinically by malabsorption and diarrhea, along with abdominal cramps, excessive gas, and bloating. The underlying molecular mechanisms of these morphological and functional derrangements remain still largely unknown.

Cytokines as regulators of intestinal epithelial cell turnover

Defining the factors and mechanisms regulating crypt cell proliferation, differentiation and enterocyte apoptosis under physiological, as well as pathological conditions, is thus critically important to improve our understanding of the pathogenesis of IBD. The **overall aim** of the present research is to identify pro-inflammatory cytokines implicated in the regulation of intestinal epithelial cell turnover. A delicate balance between growth-stimulatory and inhibitory factors is a prerequisite for normal intestinal epithelial cell turnover. There is increasing evidence that a variety of growth factors, cytokines, hormones, luminal nutrients and mesenchymal structures are essential regulators (Podolsky 1993, Ferreti et al. 1996, Ruemmele and Seidman 1998). In this study we are particularly interested in delineating the molecular mechanisms of pro-inflammatory cytokines' effects on intestinal epithelial cell turnover. Cytokines are considered as pleiotrophic, soluble factors forming an heterogeneous group of multifunctional mediators that regulate not only immunological and inflammatory processes, but also such critical cellular functions as proliferation, differentiation and apoptosis (Mosmann 1996). According to their main functions, cytokines can be grouped into pro- or anti-inflammatory cytokines, as well as chemokines, T-helper (Th)1 and Th2 cytokines. Immunocompetent cells are the major source of various cytokines. In this regard, it is important to point out the close proximity between intestinal epithelial cells and resident immunocompetent cells, present in the lamina propria and more importantly, intraepithelial lymphocytes (Figure 1). These cells, together with those aggregated in the Peyer patches and mucosal lymph nodes, form the gut associated lymphoid tissue (GALT). The latter constitutes an important source of cytokines under physiological as well as pathological conditions. In addition, there is increasing evidence

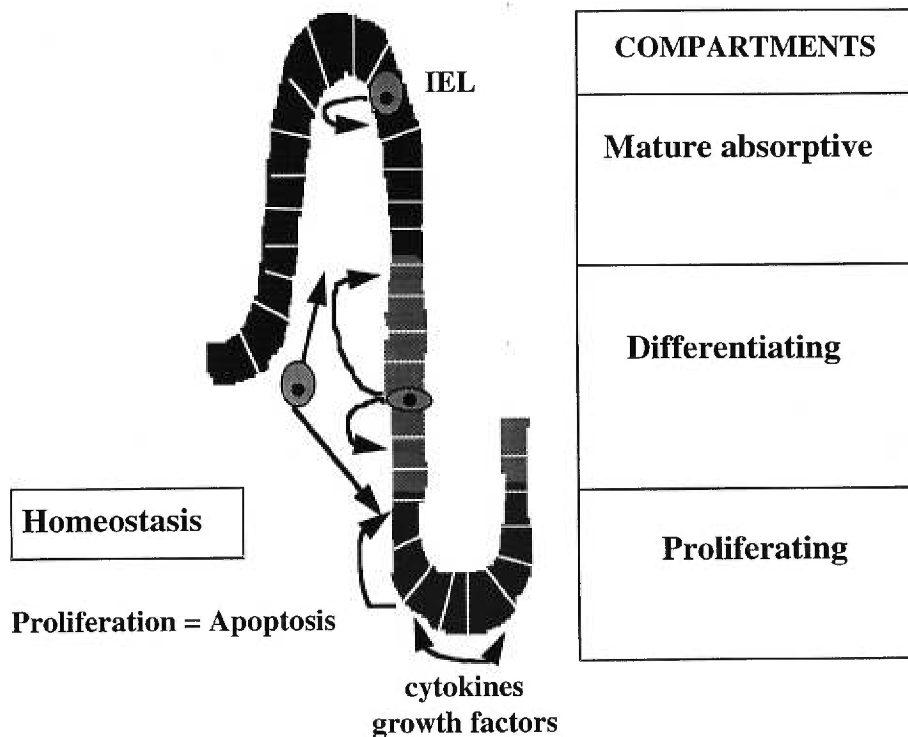

Figure 1. Normal sequence of intestinal epithelial cell turnover. Immature crypt stem cells are located in the basal crypt compartment. These cells are characterized by an extremely high proliferation rate. During their migration along the crypt-vilus axis, their daughter cells maturate to terminally differentiated enterocytes. Once reaching the villus tip, these mature, postmitotic cells die via apoptosis. This normal sequence is closely regulated by various growth factors and cytokines, produced by intrapeithelial lymphocytes (IEL), lamina propria T-cell, as well as by intestinal epithelial cells themselves, acting in an auto- or paracrine manner.

that enterocytes themselves produce and secrete important amounts of cytokines (Suemori et al. 1991, Stadnyk, 1994, Jung et al. 1995), allowing an extensive bi-directional exchange or cross-talk between epithelial and immune competent cells. A complex network of autocrine enterocyte-enterocyte and paracrine lymphocyte-enterocyte-interactions via cytokines and growth factors is considered to be of critical importance to the maintenance of homeostasis of enterocyte number and function under normal conditions (Ruemmele and Seidman, 1998).

Several studies employing high-dose irradiation or administration of cytotoxic drugs have delineated general features of the stem cell population within the intestinal crypt, responsible for the perpetual self-reconstitution (Bjerknes and Cheng 1981, Inoue et al. 1988, Gordon, 1989). Based on these studies, it was estimated that the intestinal epithelial stem cell population is composed of as few as one to four cells per crypt (Podolsky 1993). In general, stem cells are characterized by their capacity for asymmetric cell division, generating one daughter cell that replaces the parent stem cell and another daughter cell committed to progress to maturation and terminal differentiation. However, the exact nature of intestinal epithelial stem cells is relatively unclear, since these cells give raise to four distinct cell types (columnar, goblet, enteroendocrine and Paneth cells). In the search for stem cell-specific growth factors, transforming growth factor (TGF)α was found to be one of the most potent inducers of proliferation (Koyama and Podolsky, 1989, Podolsky et al. 1993, Oliver et al. 1995). This cytokine is produced in large amounts by intestinal epithelial cells themselves, suggesting both autocrine and paracrine effects on growth (Coffey et al. 1986, Suemori et al. 1991). TGFα binds to the epidermal growth factor (EGF)-receptor situated on the basal and basolateral membrane of enterocytes (Scheving et al. 1989). TGFα, which shares extensive homology to EGF and mimics its effects, is considered to be a physiological ligand for this receptor (Winkler et al. 1989). In contrast, TGFβ is a very potent inhibitor of IEC-6 (jejunal) and IEC-17 (ileal) intestinal crypt cell proliferation (Barnard et al. 1989, Kurokawa et al. 1987) and as we recently demonstrated also of human intestinal epithelial cells (Ruemmele et al. 1998). Like TGFα, it is also produced by enterocytes. Analysis of mRNA expression and protein concentration of TGFβ along the crypt-villus axis revealed a region-specific pattern, with a gradual increase from

crypt cells to maximal expression in villus enterocytes (Barnard et al. 1989). This pattern of distribution suggests that TGFβ arrests enterocyte growth in a negative feedback fashion when sufficient numbers of maturing enterocytes leave the basal proliferative compartment. The growth inhibition observed *in vitro* seems to be mediated by selective down-regulation of several genes, including c-myc and protein kinase C (Coffey et al 1988, Shipley et al. 1986, Pietenpol et al. 1990). Addition of TGFβ to crypt cell cultures led to induction of its own expression, coincident with suppression of TGFα expression, showing a self-enhancing loop of this growth inhibitory factor. TGFα mRNA expression was stimulated by its homologue EGF, with a maximal effect after 3 to 6 hours and a subsequent decline (Suemori et al. 1991). However, EGF also stimulated the expression of TGFβ which overlasted the effect of TGFα, indicating a primary self-reinforcing proliferative effect which is finally down-regulated by the induction of TGFβ (Suemori et al. 1991).

Another important trophic factor for intestinal epithelial cell growth is the keratinocyte growth factor (KGF), abundantly produced by intraepithelial γδ+T-cells (Boismenu and Havran 1994). KGF, a member of the fibroblast growth factor family, has been shown to stimulate proliferation of cells along the digestive tract from the foregut to the colon, and was useful in reducing radiation toxicity to the intestine in experimental animals (Khan et al. 1997). KGF mRNA expression has been shown to be increased in IBD (Finch et al. 1996). Taken together, these data clearly indicate that intestinal epithelial cell turnover is regulated by a variety of growth factors and cytokines.

Alterations in the local production of cytokines in immune-mediated bowel disorders, such as IBD (Sartor 1995, Fiocchi 1998), are thought to be crucial to the induction of morphological changes in the intestinal mucosa (Figure 2). These include the dramatically increased apoptosis rate, resulting in villus atrophy as well as the compensatory crypt hyperplasia (as discussed above). The ensuing paucity of mature enterocytes would result in intestinal functional derangements. There is increasing evidence that the accelerated intestinal epithelial cell turnover and the high apoptotic rate observed in these pathological conditions is mediated by cytokines released locally from infiltrating inflammatory cells recruited to the sites of active inflammation

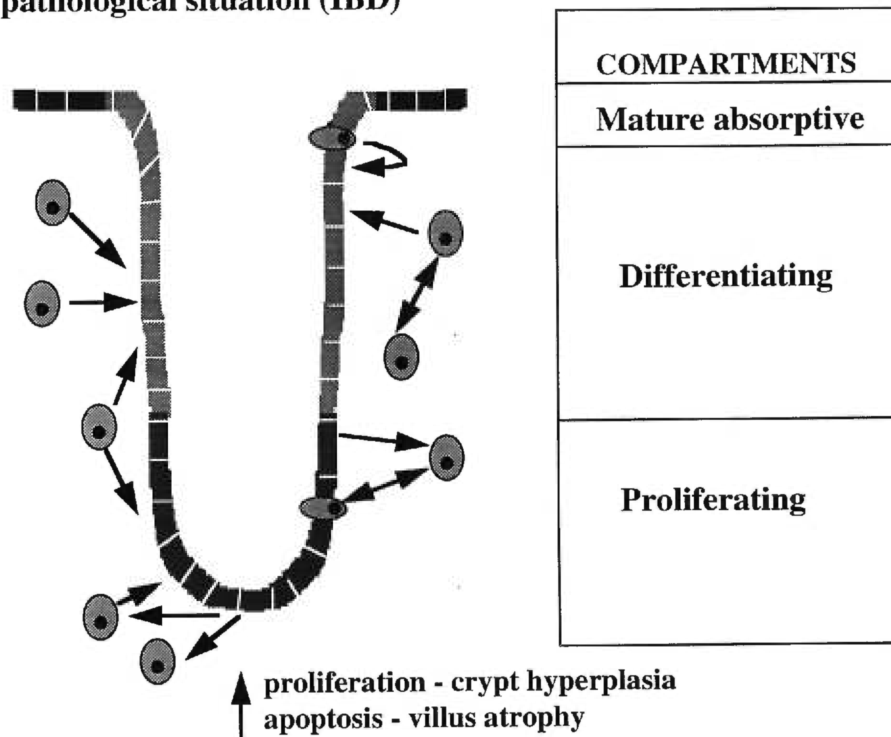

Figure 2. Altered intestinal epithelial cell turnover under pathological conditions such as immune-mediated bowel disorders. Enhanced, premature apoptotic epithelial cell death results in a paucity of mature enterocytes, leading to villus atrophy and compensatory crypt hyperplasia. Infiltrating, activated immune-competent cells contribute to this perturbation of the normal intestinal epithelial cell turnover via various pro-inflammatory cytokines such as IFNγ or TNFα. Intestinal epithelial cells are believed to potentially maintain and enhance this inflammatory reaction via the production and secretion of cytokines, which recruit and activate immune competent cells.

(Fiocchi 1998, Baumgart et al. 1998, Ruemmele and Seidman 1998). In this study, we were particularly interested in identifying pro-inflammatory factors, present in IBD tissues, which contribute to the alteration of intestinal epithelial cell turnover. In a second step, we aimed to analyze the underlying molecular mechanisms of these cytokine effects.

Only scant information exists on the physiological induction of apoptosis once a mature enterocyte/colonocyte reaches the villus tip/crypt summit. A current hypothesis is that factors implicated in the maturation and differentiation process of intestinal epithelial cells also activate the ultimate cell death machinery. TGFβ, known to be implicated in enterocyte maturation (Kurokawa et al. 1987) was proposed as one factor with such properties. Gastric and colonic epithelial cells (VACO-330) of cancer origin rapidly underwent apoptosis when stimulated with TGFβ, supporting this hypothesis (Yanagihara et al. 1992, Wang et al. 1995). As discussed above, the concentration of TGFβ along the crypt-villus axis gradually increases from the crypt compartment towards the villus tip (Barnard et al. 1989). Therefore, it is tempting to speculate about the physiological role of TGFβ, asides from its anti-proliferative properties, in triggering apoptosis of mature enterocytes. The factors and mechanisms that couple immature crypt cell proliferation with mature intestinal epithelial cell apoptosis remain largely unknown and are the subject of this research.

Molecular mechanisms of apoptosis

Apoptosis (from Greek for "falling off" of leaves), also called programmed cell death (PCD), is considered an important physiological counterpart to mitosis. The delicate, highly regulated balance between mitosis and apoptosis guarantees homeostasis in tissues with a high turnover rate, such as the intestinal epithelium. Research over the past few years has allowed us to gain insight into the complex pathways involved in cell death, yet present in an inactivated state within each cell (Green 1998). Upon receiving a specific signal, a cell can commit suicide within minutes to hours, indicating a preformed executioner-pathway. Therefore, an efficient control-apparatus must exist to suppress unwanted or untimely activation of this pathway.

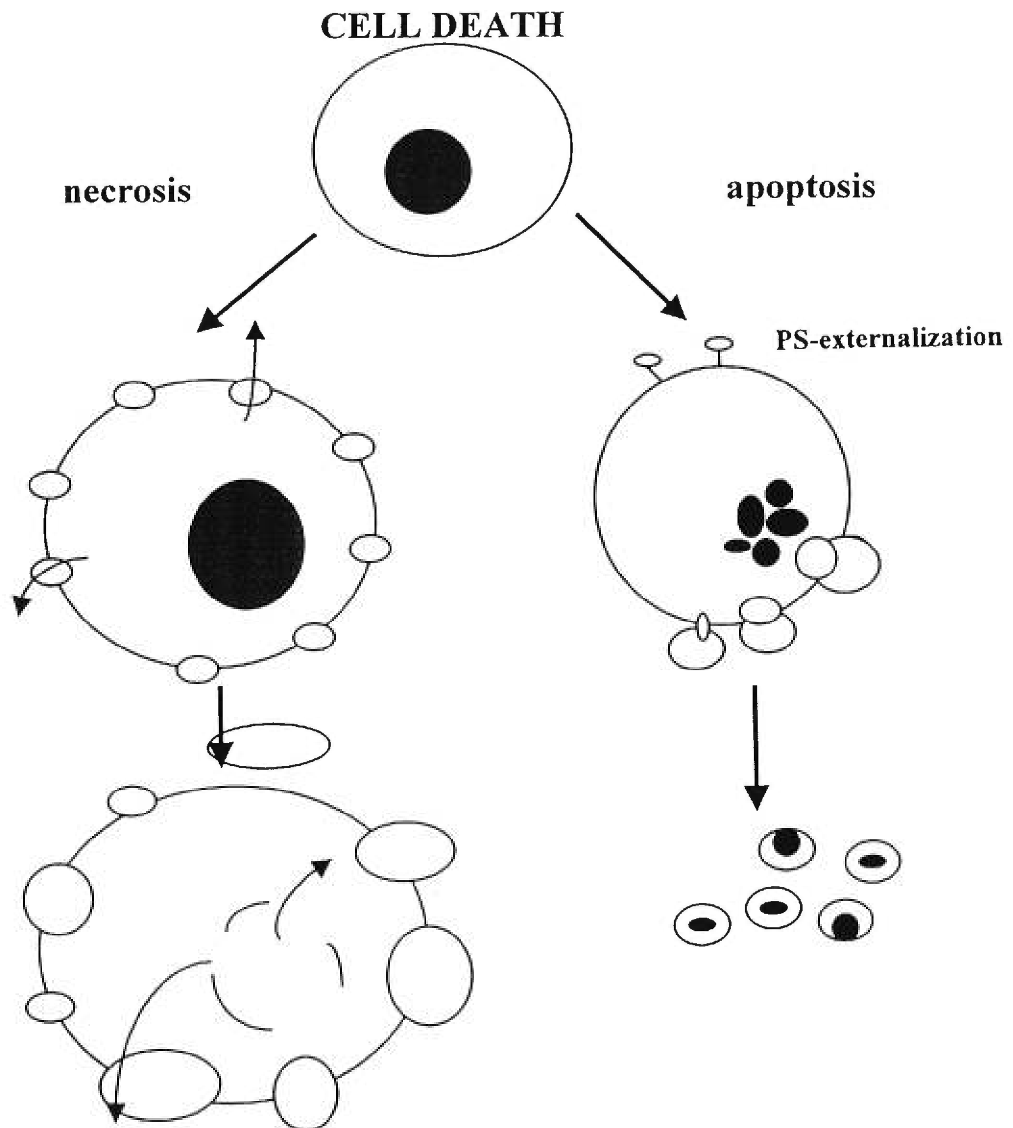

Figure 3. Apoptotic versus necrotic cell death. Apoptosis is an energy-dependent silent form of cell death that does not create an inflammatory reaction in the surrounding tissue, in contrast to necrosis. During apoptosis, the cell membrane remains longtime intact. However, very early at the onset of apoptosis it characteristically loses its asymmetry with exposure of phosphatidyl serine (PS) on the outer leaflet of the plasmamembrane. Further morphological characteristics of apoptosis are nuclear condensation and fragmentation leading to the formation of apoptotic bodies. During necrosis, the energy depleted cell swells until the membrane ruptures and the whole cytoplasmic content leaks into the surrounding tissue, leading to an inflammatory reaction.

Apoptotic cell death, in contrast to necrosis, does not provoke an inflammatory reaction in the surrounding tissue (Figure 3). Thus, the cell membrane remains intact, avoiding leakage of potently inflammatogenic cytoplasmic enzymes and proteins, such as proteases, ribonuclease etc. However, apoptotic cells are characterized by the loss of their cell membrane asymmetry, with the appearance of phosphatidyl serine (PS), normally located at the inner leaflet of the plasma membrane, on the outer surface (Fadok et al. 1992). This externalization of PS, triggered by a specific flippase, is an important signal for macrophages and various other cells to eliminate these early apoptotic cells by phagocytosis - a process which takes only a few hours. A further characteristic of apoptosis is the condensation and fragmentation of the cellular DNA with the appearance of 180 base-pairs fragments and their multiples, resulting in a characteristic DNA-ladder upon electrophoresis (Kerr et al. 1982). This degradation of the DNA is the basis for the formation of so-called apoptotic bodies. The understanding of the molecular mechanisms underlying these morphological changes and the intracellular apoptotic machinery is a field of active investigation. Scant information exists on apoptotic cell death of intestinal epithelial cells. A specific aim of this research is to analyze, if similar apoptosis-specific morphological changes can be observed in the human intestinal epithelium, especially under inflammatory conditions.

One important signalling pathway of apoptotic cell death is the intracellular caspase-cascade (Nagata 1997, Green 1998). This cascade consists of at least 10 different proteases, now refereed to as **caspases** for **c**ysteyl-prote**ases** which recognize **asp**artate in position P1 at their cleaving site. Caspases are constitutively present in the cytosol as zymogens, single chain pro-enzymes (Green, 1998). In response to various pro-apoptotic stimuli, specific caspases are activated to become fully functional proteases, first by a proteolytic cleavage to divide the single chain into a large and a small subunit and a second cleavage to remove the prodomain (N-terminal). The subunits assemble into tetramers with two active sites (Green, 1998). This proteolytic activation of caspases occurs in a cascade-like fashion, whereby these proteases serve as one another's or autocatalytically as their own substrate, thereby allowing efficient propagation and enhancement of pro-apoptotic stimuli. In a final step, several key substrates are proteolytically cleaved, resulting in definitive apoptotic cell death. This

multistep signalling pathway allows close control and regulation (Thornberry and Lazebnik 1998).

Initially, caspases were divided into 3 different sub-groups: the ICE-caspases (caspase-1,-4,-5), CPP-32-like caspases (caspase-3,-6,-7,-8,-9,-10) and ICH-1 caspases (caspases-2,-9) (Alnemiri et al. 1996). Recently, a new model was proposed (Thornberry and Lazebnik 1998) dividing caspases into initiator proteases, such as caspases-8 or -9, while others, such as caspases-3, -6 or -7, act as potent effectors of apoptosis. This latter group of caspases is responsible for the degradation of various enzymes and structural proteins involved in apoptotic cell death (Salvesen and Dixit 1997). To date, at least 30 different substrates (Cryns and Yuan 1998) of effector caspases, essentially caspases-3 and -6, are known, such as the DNA fragmentation factor 45 (DFF-45) (Liu et al. 1997), the DNA repair enzymes poly-(ADP ribosyl) polymerase (PARP) (Casciola-Rosen et al. 1996) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the actin-regulatory protein gelsolin (Kothakota et al. 1997), different structural proteins, such as α-fodrin and lamin A (Martin et al. 1995, Cryns et al. 1996), the signalling enzymes protein kinase C delta (PKCdelta), cytosolic phospholipase A2 (CPLA2) and p21-activated kinase 2 (PAK2) (Rudel and Bokoch 1997, Porter et al. 1997, Villa et al. 1997). Although it is very likely that the selective cutting of these and other key proteins is detrimental to the cell, it is unclear which of these substrates must be cleaved to commit the cell to die by apoptosis. Using DFF45 mutant mice, Zhang et al. (1998) recently demonstrated that this factor is critical to internucleosomal DNA fragmentation, the hallmark of apoptotic cell death. DFF-45 forms a heterodimer with DFF-40, a very potent DNase, thereby inactivating this nuclease. Caspase-3-mediated cleavage of the DNA fragmentation factor (DFF)-45 leads to release and induction of DFF-40 nuclease activity, resulting in characteristic chromatin condensation and DNA fragmentation (Boldin et al. 1996). However, DFF-45 mutant mice, resistant to DNA fragmentation and condensation in response to apoptotic stimuli, showed no developmental abnormality, even in organs, such as the thymus and the immune system, where apoptotic cell death is indispensable (Zhang et al. 1998). This raises the critical question of whether this endogenous system of DNA removal is essential or not for normal development and tissue homeostasis. Most of the knowledge about the caspase-cascade and their substrates are based on studies with cells of the hematopoeitic system,

such as lymphocytes, thymocytes or lymphoma cells. It is very likely, that similar events occur during apoptosis of intestinal epithelial cells. However, to date, no published study analyzed the molecular sequence of cytokine-induced apoptosis in non-transformed intestinal epithelial cells, our focus of interest.

There is increasing evidence that **mitochondria** also play an important role in apoptotic signalling, presumably in a caspase-dependent and caspase-independant way (Xiang et al. 1996, Green and Reed 1998, Adams and Cory 1998). UV- or γ-irradiation as well as dexamethasone induce thymocyte or lymphocyte apoptosis via this mitochondrial pathway (Green and Reed 1998). Recent data suggest that the release of mitochondrial cytochrome-c into the cytoplasm, allowing the interaction with Apaf-1 and caspase-9, is an important pro-apoptotic mechanism in immune competent cells (Jurgensmeier et al 1998, Yoshida et al. 1998). Cytochrome-c, Apaf-1 and caspase-9 form a complex, the so-called **apoptosom,** which allows the transactivation of inactive pro-caspase-9 to its active form, leading to the proteolytical activation of downstream effector-caspases (Zou et al. 1997, Li et al. 1997, Kroemer et al. 1997). This pathway is closely regulated by several bcl-2 gene family products, which are, with the exemption of bax, integral membrane proteins in mitochondria, endoplasmatic reticulum, or the nuclear envelope (Green 1997). These proteins form heterodimers of an anti- and pro-apoptotic member, thereby titrating one another's function. Bcl-2 and bcl-xl are known to potently inhibit the release of mitochondrial cytochrome-c, whereas bax, bak or bcl-xs are important pro-apoptotic molecules (Adams and Cory 1998). It was shown that bax directly acts on the mitochondria via binding to the permeability transition pore complex, leading to increased mitochondrial membrane permeability (Marzo et al. 1998). Three-dimensional analysis revealed that some bcl-2 family members form ion channels upon homodimerisation (Schendel et al. 1997, Minn et al. 1997, Antonsson et al. 1997), potentially changing the function of mitochondria, critical to the generation of energy. The idea has been fostered that these pores produce a leakage of the outer membrane, allowing the exit of cytochrome-c or apoptosis-inducing factor (AIF) (Jurgensmeier et al. 1998). This protein directly activates purified pro-caspase-3 in vitro, which is potently inhibited by ICE-specific caspase-inhibitor. These observations raise the possibility of a new caspase (AIF and/or others ?), located within the mitochondria (Green and Reed 1998).

Molecular mechanisms of cell cycle progression

Over the last few years, major advances were made in the understanding of the molecular mechanisms regulating the progression of a cell through the different phases of the cell cycle. The normal sequence G_0/G_1, entry into the S-phase and completion of mitosis in the G_2/M-phase is controlled by a great number of regulatory genes. Recent research revealed, that the progression of cells through the cell cycle is governed by the sequentiel formation and degradation of a series of cyclins that complex with and thereby activate several cyclin-dependent kinases (cdk), a family of 7 different serine-threonine kinases. At least 11 distinct cyclin genes in the human genome are known which can bind to a specific subunit of cdks. According to their function, these cyclins can be divided into three different categories: G_1-phase cyclins (C, D1-3, E, G, and H), S-phase cyclins (A and F) and G_2/M-phase cyclins (A and B1-2) (Pines and Hunter 1994). Cyclin D1, the first completely cloned gene of this family, plays an important role in normal cell proliferation. Inhibition of this specific cyclin with neutralizing antibodies results in cell cycle arrest in the G_1-phase (Baldin et al. 1993). It was therefore speculated that altered cyclin D1 expression is implicated in the pathogenesis of tumors. In fact, Arber et al. (1996) recently showed that increased expression of cyclin D1 is an early event in colorectal carcinogenesis. Furthermore, overexpression of cyclin D1 significantly shortens the G_1-phase of the cell cycle and makes cultured cells less dependent on exogenous growth factors (Jiang et al. 1993, Quelle et al. 1993).

The association of cyclin D1 to cdk4 or cdk6 allows the transition from G_0 to G_1. A potential downstream target of the cyclin D-cdk complex is the retinoblastoma tumor suppressor gene product pRb. During G_1, p105-Rb, in its hypophosphorylated form, neutralizes the action of the transcription factor E2F, which controls the expression of several regulatory proteins during the S-phase of the cell cycle (Blenis et al. 1993). However, phosphorylation of rb-p105 by cyclin D-cdk4/6 results in the liberation of these transcription factors thereby allowing the synthesis of the DNA-polymerase α, c-myc and serveral enzymes implicated in the biosynthesis of nucleotides. This crucial step enables the entry of a cell into the S-phase of the cell cycle. Finally, the complex of cyclin A-cdk2 phosphorylates these transcription factors (E2F, DP1 etc.) which markedly reduces their ability to bind to the DNA (LaThangue et al.1994). pRb consists

of a family of three different so-called pocket proteins: the forms p105, p107, p130. The pRb-isoforms p107, p130, act in a similar way to p105, each neutralizing specific members of the E2F transcription factor family probably implicated earlier in the G_1 phase (Cobrinik et al. 1993).

The activity of cdks is regulated by a complex control system: The cdks can be activated by phosphorylation at the specific site threonine 160/161. This reaction is catalyzed by a cdk activating kinase (CAK) (Gu et al. 1992). In contrast, phorsphorylation at the sites threonine 14 and tyrosine 15 inhibit cdk activity. Specific dephosphorylation at these sites by the phosphatase cdc25 activates thus the cdks (Jinno et al. 1994). In addition, a more recently discovered mechanism is the regulation by the association of the cyclin-cdk complex with cyclin-dependent kinase inhibitors (CKI) (Sherr and Roberts 1995). According to their mechanism of inhibition, the CKI are classed into two categories: the INK-family (inhibitory kinase): p15, p16, p18, 19 and the Cip or Kip-family (cdk or kinase inhibitory protein): p21, p27, p57. The INK complex specifically with cdk4 and 6 thereby inhibiting their activity. This blocks a cell in the G_1-phase of the cell cycle. On the other hand, the Cips block mainly the G_1-to S-phase transition by inhibiting the activity of cyclin D-cdk4/6, cyclin E-cdk2 and cyclin A-cdk2. This important control mechanism (also called checkpoint) is directly regulated by p53. In response to some forms of DNA damage (irradtiation, UV etc.), p53 is activated and turns on the transcription of p21 (El-Deiry et al. 1993). This results in a block of the cell in the late G_1-phase, allowing DNA-repair. If this repair mechanism is successfully completed, transition into the S-phase is possible. Otherwise, this cell is committed to undergo apoptosis (Levine 1997). It is important to note, that more than 50% of human cancers have a mutated p53 gene. If this checkpoint is not fully functional, cells with damaged DNA are not eliminated by apoptosis, progress in the cell cycle and finally undergo mitosis. This is a key step in tumorigenesis.

Mitogenic growth factors initiate a cascade of events during the G_1-phase via specific signalling pathways promoting cell cycle progression. One important mechanism is by enhancing the assembly of cyclin-cdk complex formation and kinase activities, as demonstrated for EGF. On the other hand, the growth inhibitory effect of TGFβ was shown to be mediated by induction of the CKIs p15 and/or p27 (Hannon and Beach, 1994).

TNFα signalling mechanisms

1) Apoptosis pathways

TNFα is thought to play a key role in the induction of apoptosis. This cytokine binds to two different membrane receptors, the p55 and p75 TNF-receptor (TNF-R) expressed on virtually all cells. A steadily increasing number of type I membrane receptors homologous to these TNF-R has been described, forming the so-called TNF-receptor superfamily, characterized by 2-6 repeats of a cysteine-rich extracellular subdomain (Ashkenazi and Dixit 1998, Nagata 1997). This receptor-family comprises, apart from the p55 and p75 TNF-R, FAS (CD95, or APO-1), the Nerve Growth Factor-receptor, along with CD 27, CD 30 and CD40 (Itoh et al. 1991, Nagata and Golstein 1995). In contrast to the extracellular homology, the intracellular domain of these receptors is different, with exception of the so-called death-receptors. This subgroup, including p55 TNF-R and FAS, is characterized by a similar intracellular sequence, the death domain (DD), enabling them to engage the cell's death machinery. Upon stimulation with the specific ligand, these receptors form trimer-complexes (Smith et al. 1994, Gruss and Dower 1995) allowing the recruitment of specific intracellular adapter molecules to the cytoplasmic DD of these receptor (Hsu et al. 1995). The p55 TNF-R binds an adapter termed TRADD (TNF-R associated death domain) through its own DD to the clustered receptor DD (Figure 4) (Hsu et al. 1995). Subsequently, TRADD functions as a platform adapter allowing binding to several signalling molecules (Rothe et al. 1995, Hsu et al. 1995, Hsu et al. 1996, Ting et al. 1996, Chinnaiyan et al. 1996). Using HeLa or murine NIH 3T3 cells with deletion mutants of FADD, it was demonstrated that binding of FADD to TRADD is essential to activate the intracellular apoptosis pathway (Hsu et al. 1996, Chinnaiyan et al. 1996, Varfolomeev et al. 1996). The p55 TNF-R-trimer-TRADD-FADD-caspase-8 complex, also called death inducing signalling complex (**DISC**), results in the activation of caspase-8 through self-cleavage, thereby initiating the caspase-cascade, and ultimately apoptosis (Ashkenazi and Dixit 1998). Given the importance of the pro-inflammatory cytokines TNFα in the pathogenesis of IBD, we speculate that similar events occur during enterocyte apoptosis in response to TNFα. In the present study, we were particularly interested in the

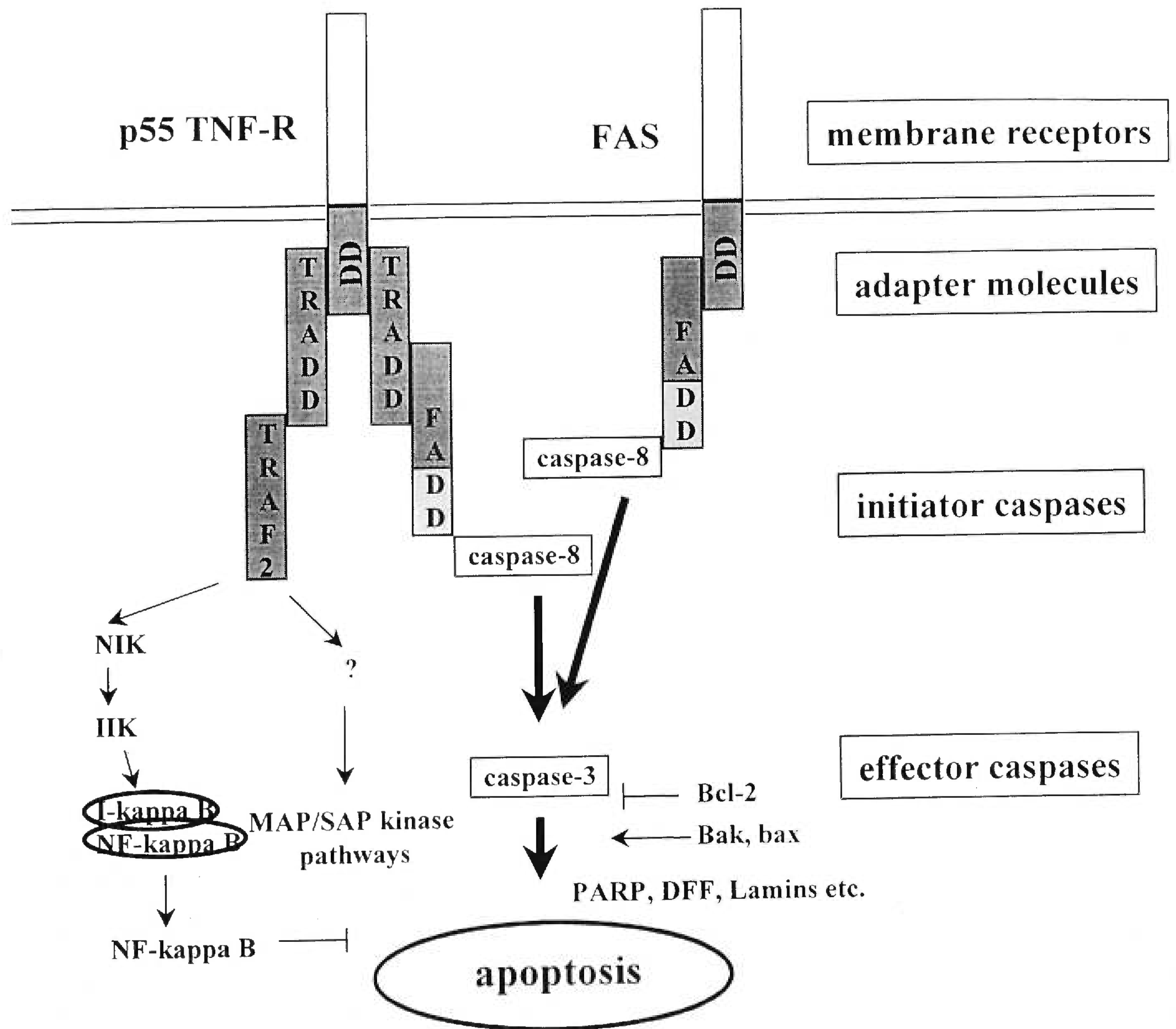

Figure 4. Schematic overview on the role of adapter molecules in the signalling of the death receptors p55 TNF-R and FAS. The receptor's cytoplasmic death domain (DD) binds intracellular adapter molecules, also characterized by a DD, such as TRADD (TNF-R associated death domain) or FADD (Fas-associated death domain). TRADD serves as an adapter platform for various other molecules. FADD is necessary to trigger apoptosis in response to the p55 TNF-R as well as FAS via the classical caspase-cascade. In contrast, binding of other molecules such as TRAF-2 (TNF-R associated factor-2) allows activation of the nuclear factor-kappa B (NF-kappaB) pathway or the MAP/SAP kinase pathway. NIK: nuclear factor-kappa B (NF-kappa B) kinase, IKK: inhibitor of kappa B kinase.

activation of specific caspases in response to stimulation of the p55 and p75 TNF-R in non-transformed enterocytes.

FAS triggers apoptosis in a very similar way to TNFα (Nagata 1997). Upon stimulation with FAS ligand, Fas-associated death domain (FADD) binds to the receptor-DD, allowing the formation of a DISC together with caspase-8. This activated caspase acts on caspases-9, -3 and others. Studies using FADD gene knockout mice confirmed the essentiality of FADD to FAS and p55 TNF-R mediated apoptosis (Yeh et al. 1998, Zhang et al. 1998).

2) Nuclear factor kappa-B pathway

TRADD can act as an adapter-platform for activated p55 TNF-R. Besides FADD, TNF-R associated factor-2 (TRAF2) and receptor-interacting protein (RIP) are also important signalling molecules of this receptor (Figure 4) (Rothe et al. 1995, Hsu et al. 1996, Varfolomeev et al. 1996). Recently, evidence was put forward showing that TNFα activates the nuclear factor-kappa B (NF-kappa B) kinase (NIK), an important regulator of the generation of pro-inflammatory responses, via TRAF-2 and RIP (Malini et al. 1997, Regnier et al. 1997, DiDonato et al. 1997, Mercurio et al. 1997, Woronicz et al. 1997). However, recent studies in a TRAF-2 knock out mouse model showed only slightly impaired NF-kappa B response to TNFα, suggesting that adapter molecules in addition to TRAF-2 are involved in this signal transmission (Yeh et al. 1997, Lee et al. 1997). NIK in turn activates the inhibitor of kappa B (I-kappa B) kinase complex, resulting in phosphorylation of I-kappa B and subsequently, ubiquination and proteolytic degradation. Normally, NF-kappa B is bound in the cytoplasm to I-kappa B, thereby forming a heterodimer. Upon degradation of I-kappa B, the two subunits of NF-kappa B (p50 and p65) are released and move to the nucleus to activate transcription of various genes involved in inflammatory processes, such as the cytokines IL-1β and IL-6, or the chemokines IL-8, macrophage colony-stimulating factor, monocyte chemotactic protein 1 or intercellular adhesion molecule 1 (ICAM-1) (Lenardo and Baltimore 1989, Baeuerle 1991). Furthermore, the production of TNFα is also activated by NF-kappa B, indicating a potent positive autoregulatory loop. Cells lacking one component of NF-kappa B or expressing I-kappa B mutants, which cannot be phosphorylated and

ubiquinated, were found to be more sensitive to TNF-induced apoptosis. This suggests that one of the target genes of NF-kappa B encodes for an anti-apoptotic survival factor (Beg and Baltimore 1996, Liu et al. 1996, Van Antwerp et al. 1996, Wang et al. 1996). In fact, TNF-induced cytotoxicity is significantly enhanced in the presence of protein-synthesis inhibitors, such as cycloheximide or actinomycin D. This effect might be due to the inhibition of NF-kappa B induced gene-expression. Furthermore, FAS, which does not activate NF-kappa B, triggers a stronger apoptotic response than TNFα, which stimulates both the apoptotic cascade and NF-kappa B (Nagata 1997).

3) MAP-SAP Kinase pathways

Other important signalling pathways via the TNF-R, activated via TRADD-TRAF-2/RIP, include the intracellular cascade of mitogen-activated protein (MAP) kinases and stress-activated protein (SAP) kinases (Figure 5). These signal-transducing protein phosphorylation cascades are involved in several important cellular functions, such as the regulation of specific transcription factors implicated in cell cycle progression and mitosis, the arrangement of the cytoskeleton, regulation of differentiation, maturation, etc (Denhardt 1996, Seger and Krebs 1995). The intracellular signalling pathways, connecting stimulation of specific cell surface receptors (mainly cytokine and growth factor receptors) to cytoplasmic and ultimately nuclear processes, can be separated into **p42 and p44-MAP kinases** (Erk1/2), and **SAP kinases**, comprising **p38** and c-**jun N**-terminal **k**inase (**p-46 and p55-JNK**). Research over the past few years has unraveled the complex signalling hierarchy of these cascades, however, only little information exists on the signalling events in response to proinflammatory cytokines in intestinal epithelial cells. Each enzyme of this cascade, characterized by a serine-threonine kinase activity, is activated via dual-phosphorylation on tyrosine and threonine residues, and therefore serves as a substrate for kinases located upstream (Matsuda et al. 1992, Yashar et al. 1993). The cascade-like activation system allows potent amplification and specificity of the transmitted signal. Recently, it was suggested (Seger and Krebs, 1995) that the **MAP kinases Erk1/2**, also called p42/44, are central components of the **MAP kinase-cascade**. These proline-directed protein kinases, located both in the cytoplasm and the nucleus, preferentially phosphorylate serine or threonine residues next to proline with the most stringent consensus sequence Pro-Leu-Ser/Thr-Pro (Gonzalez et al. 1991). Their major substrates

are transcription factors or nuclear proteins, such as c-myc, c-myb, p53, Elk-1 or c-fos (Angel and Karin 1991, Chen et al. 1993, Chuand and Ng 1994, Cavigelli et al. 1995, Treisman 1995), which together with c-jun, form the dimer activating protein 1 (AP-1). In addition, the MAP Kinases Erk1/2 also phosphorylate cytoskeleton elements, such as MAP-1, MAP-2, MAP-4, TAU and others, thereby regulating cytoskeleton rearrangements and cellular morphology (Minshull et al. 1994). The upstream events, leading to phosphorylation of Erk1/2, comprise at least 3 consecutively activated kinases. In response to TNFα, Erk1/2 is thought to be mainly activated via Raf-1 kinase. However, Raf-1 kinase independent stimulation was also described in some cells (Dent et al. 1992). In this work, we were particularly interested in unraveling the signalling mechanisms of TNFα in immature crypt cell growth.

Similar to the MAP kinases Erk1/2, the **SAP kinases p38** and **JNK-p46/54** are also activated by dual-phosphorylation in a cascade-like fashion (Davis 1994). Highly specific SAP kinase kinases (SAPKK) phosphorylate JNK at the sequence Thr-Pro-Tyr, whereas p38 is phosphorylated at Thr-Glu-Tyr (Raingeaud et al. 1995, Doza et al. 1995). To date, 5 different SAPKK are known, each with different affinity for the two pathways p38 and JNK, indicating important interactions between these two signal transduction pathways (Paul et al. 1997). At the SAPKK level, no interference with the MAP kinase cascade occurs. In contrast, just one level upstream, the kinases which activate SAPKK termed MEKK 1-5, can also stimulate the MAP kinase pathway. MEKK 2 and 3 primarily phosphorylate different kinases of the JNK and simultaneously the MAP Kinase cascade, whereas MEKK1 and 5 activate JNK exclusively (Paul et al. 1997). The p38 pathway is not activated by MEKKs. However, recently with TAK-1 (transforming growth factor β-activated kinase) a MEKK homologue protein was described, which is able to activate p38 (Moriguchi et al. 1996). Substrates of SAP kinases include several transcription factors, such as activated transcription factor-2 (ATF-2), Elk-1 or CREB (cAMP-responsive element B) (Gupta et al. 1995, Livingstone et al. 1995, Van Dam et al. 1995). These factors form homodimers, allowing binding to the promotor/enhancer region of specific genes. The most relevant substrate of JNK is c-jun, activated by its phosphorylation at positions Ser 63 and Ser 73 (Karin 1995).

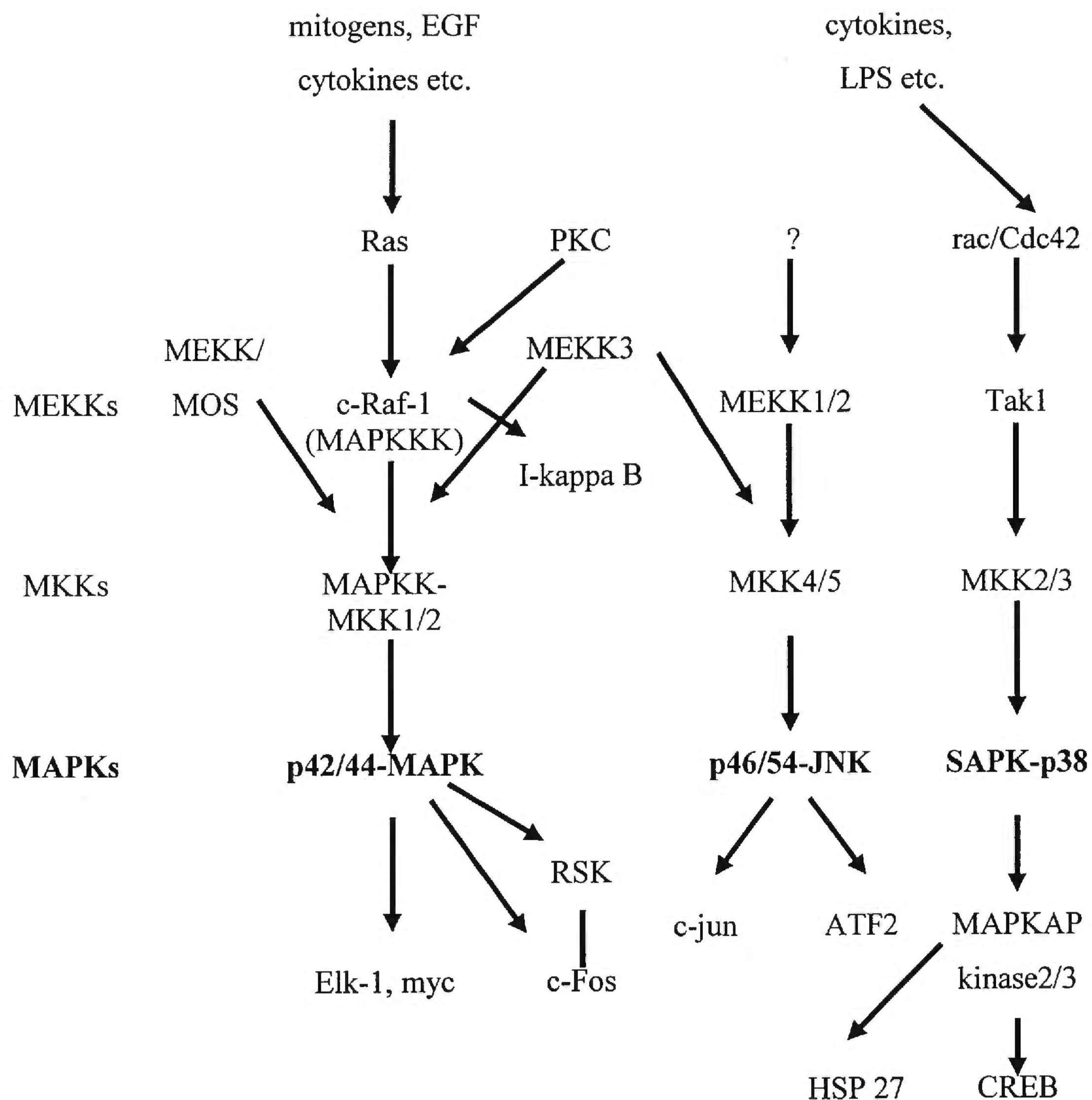

Figure 5. Schematic overview of the mitogen-activated protein (MAP) kinases and stress-activated protein (SAP) kinases-cascade. This intracellular signalling cascade is implicated in cell growth, differentiation as well as cell death. Each kinase is activated via dual-phosphorylation on tyrosine and threonine residues, and therefore serves as a substrate for kinases located upstream.

Phosphorylated c-jun binds to activated c-fos forming the AP-1 complex (Smeal et al. 1989), known to induce transcription of a variety of proto-oncogenes, including recently discovered genes also involved in apoptosis. It was suggested that one possible AP-1 target could be the FAS gene, which includes a typical AP-1 response element (TGAGTAA) at position -552 to -449 (Colotta et al. 1992, Cheng et al. 1995). Another potential target gene might be the FAS ligand, since its promotor also contains an AP-1 response element (Takahasi et al. 1994).

II. AIMS

There is increasing evidence that cytokines and growth factors are important physiological regulators of intestinal epithelial cell turnover (Podolsky 1993). Any perturbations in the closely controlled production and secretion of cytokines in the complex network of the intestinal mucosa, such as occurs in the presence of inflammation, potentially alter the rate of enterocyte turnover, leading to important morphological and functional derangements of the intestine (Ruemmele and Seidman 1998). In pathological conditions such as IBD, a markedly increased enterocyte apoptosis rate along with crypt cell hyperplasia was noted in the inflamed mucosa, concomitant with infiltrates of activated immune cells (Moss et al. 1996, Strater et al. 1997). In zones of active inflammation, excessive amounts of TNFα and IFNγ are released by activated immune-cells (Braegger et al. 1992, Fais et al. 1995, Dionne et al. 1998). We speculate therefore that these pro-inflammatory cytokines play a critical role in the pathogenesis of IBD. However, their effects and precise molecular mechanisms on intestinal epithelial cells remain largely unknown. Our **hypothesis** is that IFNγ and TNFα act directly on intestinal epithelial cells leading to altered proliferation rate and inducing apoptosis.

Therefore, the aims of this research were

- to specifically identify the role of TNFα and/or IFNγ as regulators of intestinal epithelial cell turnover (proliferation and apoptosis/necrosis)
- to analyze the signal transduction pathways involved in cytokine-induced enterocyte growth alterations.
- to analyze the molecular mechanisms of enterocyte apoptosis, including the receptors and signaling mechanisms involved.

To address these specific points, four different, complementary experimental models of intestinal epithelial cells were employed:

- **HIEC** - human intestinal epithelial cell line of fetal ileal origin, as a model of human non-transformed immature intestinal crypt epithelial cells (Perrault and Beaulieu, 1996).
- **IEC-6** cell line of rat jejunal origin, as a model of non-transformed immature intestinal crypt epithelial cells from a mature animal (Quaroni et al. 1989).
- **Caco-2** cell line of human colon cancer origin, as a model of partially differentiated human enterocytes.
- ***ex vivo* human colon explant cultures,** as a model recapitulating the *in vivo* situation, preserving the complex network of interactions between the various cell populations within the intestinal mucosa, including the matrix.

The **role of TNFα and/or IFNγ as regulators of intestinal epithelial cell turnover** was addressed in the following publication:

#1) **Ruemmele FM**, Gurbindo C, Mansour AM, et al. Effect of interferon gamma on growth, apoptosis and MHC class II expression of immature intestinal crypt (IEC-6) cells. *J Cell Physiol* 1998;176:120-126.

This paper elaborates the effects and signaling hierarchy of IFNγ and TNFα on immature intestinal crypt cells. IFNγ arrested IEC-6 cells in the G_0/G_1-phase of the cell cycle potently inhibiting their growth, without inducing apoptosis or necrosis. On the other hand, TNFα markedly stimulated IEC-6 growth. However, when added simultaneously, IFNγ completely blocked the effect of TNFα, even at very low doses, indicating a clear signaling hierarchy. Furthermore, induction and up-regulation of MHC class II glycoprotein expression on IEC-6 cells by IFNγ and TNFα was analyzed, showing a synergistic effect between these two cytokines.

The **molecular mechanisms of TNFα** induced growth stimulation and apoptosis in IEC-6 cells were addressed in the following papers:

#2) Dionne S, D'Agata ID, **Ruemmele FM**, et al. Tyrosine Kinase and MAPK inhibition of TNF alpha and EGF stimulated IEC-6 cell growth. *Biochem Biophys Res Commun* 1998; 242:146-150.

This work unravels the intracellular signal transduction pathways involved in TNFα and EGF growth stimulation of IEC-6 cells. The promitogenic effects of both TNFα and EGF were found to be mediated by rapid activation via phosphorylation of the MAPKinases Erk1 and Erk2. Inhibition of the MAPKinase Kinase (MKK) completely blocked phosphorylation of Erk1/2, thus abolishing the growth promotory effects of TNFα and EGF.

#3) **Ruemmele FM**, Dionne S, Levy E, Seidman EG. TNFα-induced IEC-6 cell apoptosis requires activation of ICE-caspases whereas complete inhibition of the caspase-cascade led to necrotic cell death. *Biochem Biophys Res Commun* (in press).

This study analyzes the role and underlying signaling mechanisms of TNFα in inducing immature intestinal epithelial cell death. TNFα potently induced IEC-6 cell apoptosis. Receptor analysis revealed that this effect was more than 100-fold stronger if both TNF-R were stimulated, compared to the p55 TNF-R alone. These data indicate an important apoptosis enhancing effect of the p75 TNF-R. The apoptotic signaling required activation of ICE-caspases, but was independent of caspase-3. Complete inhibition of the caspase-cascade with zVAD-fmk switched the apoptotic response to TNFα into a necrotic form of cell death, suggesting an important anti-necrotic role of caspases.

The effects of TNFα and IFNγ on the **regulation of the FAS-FAS ligand pathway in enterocyte apoptosis** was then analyzed using a non-transformed human enterocyte cell line and *ex vivo* human colonic explant cultures.

#4) **Ruemmele FM**, Russo P, Beaulieu J-F, Dionne S, Levy E, Lentze MJ, Seidman EG. Susceptibility to FAS-induced apoptosis in human non-tumoral enterocytes - the role of co-stimulatory factors. *J Cell Physiol* (in press).

This study establishes that non-transformed human intestinal epithelial cells as well as colonocytes constitutively express FAS, but that costimulatory factors are necessary to sensitize these cells to FAS-induced apoptosis. TNFα and IFNγ, alone and in combination, proved to be potent upregulators of FAS expression in a synergistic way,

dramatically increasing the susceptibility to FAS-induced apoptosis. This important sensitizing effect was confirmed in *ex vivo* colon explants using the *in situ* TUNEL assay. FAS was not observed to be involved in the regulation of normal enterocyte proliferation. No FAS ligand expression nor its induction by cytokines was seen in non-transformed human intestinal epithelial cells.

To further elucidate the intracellular signaling mechanisms of enterocyte apoptosis, butyrate-induced apoptosis was studied in the Caco-2 cell model. Dietary short chain fatty acids, such as butyrate, are known to stimulate the growth of normal colonocytes, whereas they induce apoptosis in transformed colon cancer cells.

#5) **Ruemmele FM**, Dionne S, Qureshi I, Sarma SDR, Levy E, Seidman EG. Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic bak and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). *Cell Death Diff* (in press).

In this paper, we showed that butyrate potently induced colon cancer cell apoptosis via the activation of caspase-3. This protease is responsible for the degradation of several important cellular enzymes, as exemplified by the DNA-repair enzyme poly-(ADP-ribose) polymerase (PARP). Inhibition of caspase-3 completely abolished the apoptotic effect of butyrate, confirming the importance of this pathway. Furthermore, butyrate upregulated the expression of pro-apoptotic bak, thereby activating the mitochondrial apoptosis-pathway.

JOURNAL OF CELLULAR PHYSIOLOGY 176:120–126 (1998)

Effects of Interferon γ on Growth, Apoptosis, and MHC Class II Expression of Immature Rat Intestinal Crypt (IEC-6) Cells

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Intestinal epithelial cells and the mucosal immune cells in close proximity are thought to interact very closely. One well-established mechanism of this intercellular cross-talk is via the production of cytokines such as interferon gamma (IFNγ). The aim of this study was to analyze the effects of IFNγ on intestinal crypt epithelial cells. IEC-6 cells were cultured in the presence or absence of IFNγ to measure its effects on proliferation, cell cycle, apoptosis, and major histocompatibility complex (MHC) class II antigen expression. Even at very low doses (0.01 U/ml), IFNγ significantly inhibited IEC-6 cell proliferation, as demonstrated by reduced ^{3}H-thymidine uptake, stable cell count, and complete arrest in the quiescent G_0/G_1 phase of the cell cycle. Incubation with supraphysiological doses of IFNγ (100–1,000 U/ml) did not induce apoptosis, as assessed by morphology and the TUNEL assay. IFNγ significantly induced de novo IEC-6 class II antigen expression. Tumor necrosis factor alpha (TNFα), which alone had no effect, synergistically enhanced this effect of IFNγ. MHC class II antigen expression was observed to be independent of cell cycle phase. Our results indicate that IFNγ alters immature crypt epithelial cell turnover and upregulates MHC class II expression. These alterations may be important in the pathogenesis of immune-mediated bowel disorders. J. Cell. Physiol. 176:120–126, 1998. © 1998 Wiley-Liss, Inc.

There is increasing evidence that intestinal epithelial cells are not only the target of immune reactions, but are also actively involved in modulating and maintaining the mucosal inflammatory response. This is of particular relevance to the pathogenesis of immune-mediated bowel diseases such as ulcerative colitis, Crohn's disease, and celiac disease. Mature enterocytes express major histocompatibility complex (MHC) class II glycoproteins on their surface, indicating their potential as antigen presenting cells (Mayer and Shlien, 1987; Bland and Kambarage, 1991). Immature crypt epithelial cells are class II negative in humans, and only weakly positive in rats and mice (Bland and Kambarage, 1991; Bland and Whiting, 1993). In pathological conditions where excessive antigenic stimulation and activation of mucosal immune cells are present, enterocytes have been shown to express an increased density of class II antigens. This aberrant expression extends to crypt and colonic epithelial cells (Mason et al., 1981; Arnaud-Battandier et al., 1986).

It has been suggested that cytokines, produced locally by activated inflammatory mucosal cells, are involved in the adaptive process leading to de novo class II molecule expression and simultaneously, to alterations in crypt cell renewal (Bland and Kambarage, 1991). Interferon gamma (IFNγ) plays an important role as an inducer of class II antigen expression on epithelial cells. In the T84 and HT29 transformed cell lines of colonic origin, IFNγ stimulated MHC II antigen expression (Lundin et al., 1987). Using jejunal explants cultured with IFNγ, increased enterocyte MHC class II molecules were also noted (Sturgess et al., 1992). Induction of class II molecules from conditioned medium of mitogen-stimulated lymphocytes has been demonstrated in ileal crypt cells (IEC-17; Cerf-Bensussan et al., 1984). However, the factors that modulate the expression of class II molecules in immature intestinal epithelial cells remain poorly defined.

Another important effect of IFNγ on enterocytes was recently suggested by organ culture studies in celiac disease (Przemioslo et al., 1995). Treatment of duodenal biopsies with supernatant from gliadin-sensitive T-cell clones that had been stimulated with gluten re-

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sulted in a significant reduction in enterocyte height compared to untreated controls. This effect was inhibited by anti-interferon γ antibodies, indicating the potential importance of this cytokine in the pathogenesis of the mucosal damage. Studies using T lymphocytes, cultured human salivary gland cells as well as oligodendrocytes demonstrated that IFNγ has potent antiproliferative and apoptotic effects (Vartanian et al., 1995; Sakatsume and Finbloom, 1996; Wu et al., 1996). This cytotoxic effect was also reported using transformed intestinal epithelial cells (Deem et al., 1991). Furthermore, IFNγ is known to disrupt the epithelial barrier, as shown in several electrophysiological studies on intestinal monolayer cell cultures (Madara and Stafford, 1989; Planchon et al., 1994). However, information is lacking on the effects of IFNγ on proliferation and apoptosis in nontransformed, undifferentiated intestinal crypt epithelial cells.

In the present study, the IEC-6 cell line was chosen as an in vitro model of immature crypt cells capable of self-renewal and differentiation (Quaroni et al., 1979). We studied the effect of IFNγ on IEC-6 cell proliferation, cell cycle distribution, and induction of apoptosis. To determine whether IEC-6 cells can express class II antigen on their surface, we measured basal MHC class II expression and its modulation by IFNγ and tumor necrosis factor alpha (TNFα).

MATERIALS AND METHODS

Cell cultures

IEC-6 cells (ATCC, Rockville, MD) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified eagle medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), 1 mM sodium pyruvate, 50 U/ml penicillin G, and 100 μg/ml streptomycin. Recombinant rat IFNγ was used at concentrations of 1–1,000 U/ml and monoclonal anti-rat IFNγ (neutralizing activity 6 × 10^3 U/ml) at 25–200 U/ml (both from Amgen, Thousand Oaks, CA). Human recombinant TNFα (Genzyme, St. Louis, MO), 76% homologous with murine TNFα (Marmenout et al., 1985), was used at concentrations of 0.1–100 ng/ml. This dose range was previously shown to enhance HLA molecule expression in colonic epithelial cells (Kvale et al., 1988). The concentrations of IFNγ and TNFα utilized are within the physiologic range found in the intestinal mucosa, as demonstrated by in vitro studies on T lymphocyte activation (MacDonald et al., 1990). Polyclonal rabbit anti-human TNFα (neutralizing activity 10^6 U/ml) was employed at 50 neutralizing U/ml (Genzyme). The antibody employed for MHC class II determination was fluorescein-isothiocyanate (FITC)-conjugated MRC OX6 mouse monoclonal IgG_1 (Cederlane Lab, Hornby, Ontario), at a concentration of 0.25 mg/ml in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). This antibody detects nonpolymorphic determinants on Ia molecules encoded in the I-A-equivalent region of the rat MHC (McMaster and Williams, 1979). Isotype-matched FITC-conjugated mouse IgG_1 (Cederlane) was used (0.25 mg/ml) in all experiments as a control for nonspecific fluorescence.

Proliferation assay

IEC-6 cells (6 × 10^5 cells/ml) were allowed to reach subconfluence over 48 h in 24-well plates (Falcon Plastics, Oxnard, CA). The cells were then washed with PBS and changed to FCS-free supplemented DMEM for a 24-h stabilization period. Cells were cultured in the absence (control) or presence of IFNγ for 20 h in supplemented DMEM containing only 1% FCS. Proliferation was studied by monitoring changes in DNA synthesis, as measured by ^{3}H-thymidine uptake (2 μCi/ml) added during the last 2 h of culture. Cells were trypsinized and harvested, and radioactivity was measured in trichloroacetic acid protein precipitates as counts per minute per microgram DNA. Results were expressed as percentage of ^{3}H incorporation relative to control wells without cytokine. Total DNA was determined for each well in the sonicated cells by a microfluorometric method using calf thymus DNA as the standard (Boehringer, Mannheim, Germany). Furthermore, absolute cell numbers were counted using a hematocytometer at several time intervals.

Apoptosis assays

Cells were cultured in 24-well plates as described above. To measure the cytotoxic effect of IFNγ, IEC-6 cells were treated for 24–72 h with 100 to 1,000 U/ml IFNγ. After harvesting (floating cells included), the cells were stained with the HOECHST 33342 DNA dye (5 μg/ml) (Sigma) for 5 min at room temperature and counterstained with propidium iodide (PI, 5 μg/ml; Sigma). Cells with typical nuclear fragmentation and bright HOECHST staining were considered apoptotic. A commercially available cellular DNA fragmentation kit (TUNEL assay, Boehringer) was used in parallel. Briefly, after fixation in 2% paraformaldehyde, cells were washed and incubated in 0.2% Tween-20-PBS for 2 min. Subsequently, IEC-6 cells were incubated with 50 μl of the labeling mixture consisting of FITC-labeled dUTP and terminal deoxynucleotide transferase (tdt) at 37°C for 60 min. Cells incubated with labeling mixture without tdt served as negative controls. The fluorescence of 1 × 10^4 cells was measured by flow cytometry (FACScan, Becton Dickinson, Mississauga, Ontario) and the percentage of apoptotic cells calculated. Cycloheximide-treated IEC-6 cells (30 μg/ml for 24 h) served as positive apoptotic controls.

Class II antigen induction

IEC-6 cells were cultured in 25 cm^2 flasks (Falcon) at a density of 6 × 10^5 cells/ml. After a 24-h stabilization period without FCS, the cells were incubated in complete medium (1% FCS), with or without (control cultures) various amounts of IFNγ alone, or in combination with TNFα, for different durations. Studies examining the specificity of cytokine effects were carried out by simultaneously adding the corresponding specific anti-cytokine antibody to cultures. For flow cytometric analysis, the cells were detached from the culture flasks with trypsin-EDTA, washed, and counted. Cellular viability was verified for all cultures by trypan blue exclusion.

Detection of class II antigen expression

IEC-6 cells (2 × 10^6 cells/ml) were resuspended in 10 μl of FITC-conjugated MRC OX6 mouse monoclonal IgG_1 antibody or in 10 μl mouse FITC-conjugated IgG_1 to measure isotypic background staining, and incubated for 30 min at 4°C in the dark. Unbound antibody

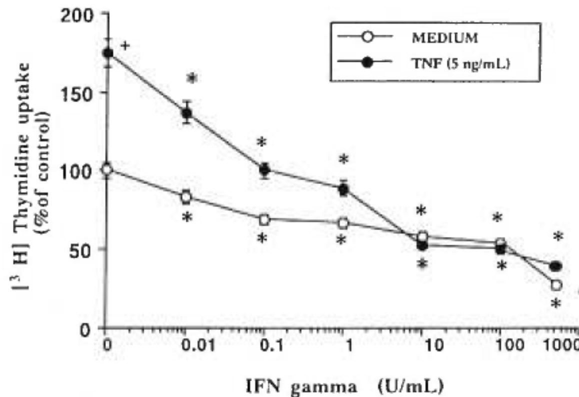

Fig. 1. Effect of IFNγ on IEC-6 cell proliferation. The cells were cultured in the presence of IFNγ alone (○) or with IFNγ and TNFα (●) at the doses indicated for 20 h. Proliferation of IEC-6 cells was measured by the incorporation of ^{3}H-labeled thymidine. Basal proliferation without cytokine addition (control wells) was set at 100%. Results are expressed as mean ± standard deviation vs. control for five experiments (in triplicate). *$P < 0.05$, $^+P < 0.005$ vs. control.

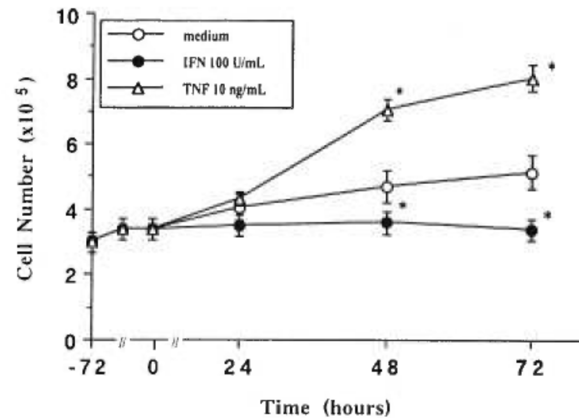

Fig. 2. Effect of IFNγ and TNFα on IEC-6 cell proliferation. The cells were cultured in the presence of IFNγ (100 U/ml), TNFα (10 ng/ml), or medium without additional cytokines. At the time intervals indicated, the absolute cell number was counted. *$P < 0.05$ vs. medium without cytokine addition.

was eliminated by washing with cold PBS-1% BSA. Thereafter, the cells were fixed for 30 min in PBS-2% paraformaldehyde. A minimum of 1×10^4 cells were analyzed by flow cytometry using Lysys II software (Becton Dickinson). Expression of class II antigens was analyzed with regard to the total percentage of positive cells over background, as well as a mathematically calculated median of fluorescence intensity, on a four-decade scale. The percentage of positive cells was calculated by integration of the area under the curve at a set threshold based on the background staining observed. Data presented are percent positive cells over this background induced by IFNγ relative to the control. The effect of IFNγ plus TNFα relative to IFNγ alone was also determined by integration of their respective areas. Each experiment was repeated at least three times.

In a separate experimental setting, MHC II antigen expression was correlated with IEC-6 cell cycle status. After incubation with the FITC-labeled anti-class II antibody (as described above), the cells were fixed in a final concentration of 0.25% paraformaldehyde for 30 min at 4°C. After permeabilization for 15 min at 37°C in 0.2% Tween 20 PBS, the cellular pellet was stained in 1 ml PBS containing 5 μg/ml PI and 65 Kunitz units RNase for 30 min. Samples were then analyzed by flow cytometry, collecting FITC signals on the logarithmic scale and PI signals on the linear scale. For these assays, control samples stained separately with anti-class II antibody or PI were prepared to verify the accuracy of the combined FITC-PI staining. For each sample, 1×10^4 cells were analyzed using the mathematical model of cell fit RFIT software (BDIS, Becton Dickinson) to determine the percentage of cells in the G_0/G_1, S, and the G_2 + M phases of the cell cycle. All samples were gated to eliminate doublets and aggregates.

Statistical analysis

Values are expressed as mean ± standard deviation. Results were compared by the two-tailed Mann-Whitney U-test and Wilcoxon signed rank test. Differences with P values < 0.05 were regarded as significant.

RESULTS

Cell proliferation and cell cycle distribution

Incubation with IFNγ resulted in an antiproliferative effect at all doses tested with a significantly reduced thymidine uptake into DNA (Fig. 1) and stable cell number (Fig. 2). Growth inhibition was maximal (70% ± 3 of controls) with 500 U/ml IFNγ ($P < 0.05$). Cell cycle analysis of IEC-6 cells showed that 24 h after serum withdrawal, 94% of the cells were in G_0/G_1, 3% in S, and 3% in G_2 + M phases. These results indicated that at this time point (0 h), IEC-6 cells were principally in the quiescent phase of the cell cycle. Control cultures in complete medium (+1% FCS) without exogenous cytokines revealed that IEC-6 cells began exiting the G_0/G_1 phase after 24 h, with an increase of cells in the S (12%) and G_2 + M phases (4%; Fig. 3). In contrast, during the same period, cells cultured in complete medium with IFNγ remained in the quiescent G_0/G_1 phase. At higher doses of IFNγ (500–1,000 U/ml), 98% of IEC-6 cells rested in the G_0/G_1 phase.

Incubation with TNFα alone promoted the entry of 21% into S and 13% into G_2 + M phases ($P < 0.001$). However, the simultaneous addition of IFNγ and TNFα did not induce the entry of IEC-6 cells into S phase. Indeed, the results for IFNγ with TNFα were similar to those observed with IFNγ alone (Fig. 3). These differences in cell cycle status tended to dissipate after 72 h of culture. Cells cultured in the presence of IFNγ remained in the quiescent phase, whereas controls or cells cultured with TNFα eventually exited the active phase, to rest in the quiescent phase. Simultaneous addition of IFNγ at various doses with TNFα (Fig. 1) revealed a hierarchy in cellular proliferation signaling with a clear, inhibitory dominance of IFNγ.

Apoptosis

In untreated control cultures, only 2.0% ± 0.5 of IEC-6 cells underwent spontaneous apoptosis. Cells treated

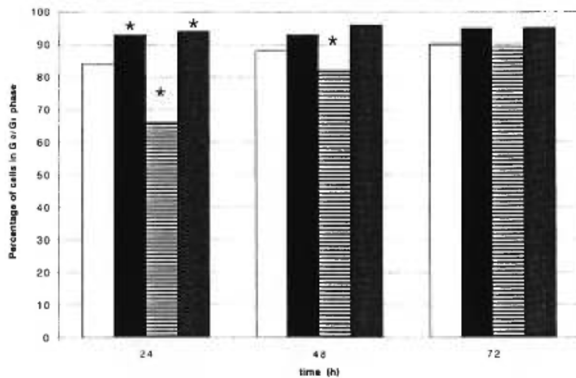

Fig. 3. Effect of IFNγ and TNFα on IEC-6 cell cycle status. The distribution of intestinal crypt cells in each phase of the cell cycle was measured by the amount of DNA using flow cytometry. The percentage of cells in the quiescent phase (G_0/G_1) compared to cells in all phases of the cell cycle is shown. The difference from 100% thus corresponds to the percentage of cells in S plus G_2 + M phases. At 0 h, 94% of IEC-6 cells were in G_0/G_1, 3% in S, and 3% in G_2 + M phases. Results are expressed as the mean of five experiments. $^*P < 0.05$ vs. control. □, control; ■, IFNγ 100 U/ml; ▤, TNFα 10 ng/ml; ▦, IFNγ and TNFα.

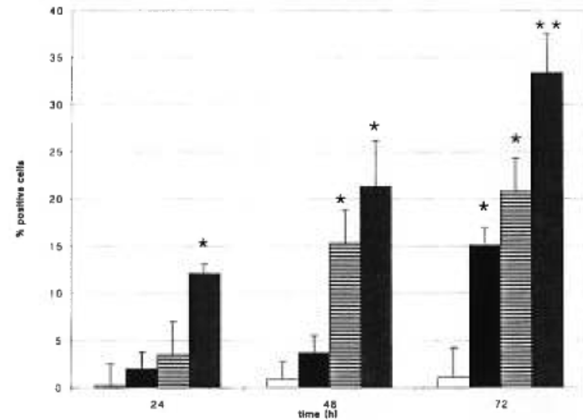

Fig. 4. Effect of IFNγ on IEC-6 cell MHC class II expression. The cells were cultured for 72 h in the presence of IFNγ (1 to 100 U/ml). Class II antigen expression was measured by flow cytometry after incubation with FITC-conjugated I-A-specific monoclonal antibody. The percentage of positive cells over background is represented by the histograms. Results shown are the mean of at least five experiments. $^*P < 0.05$ vs. control (at 24 h). $^{**}P < 0.05 - P < 0.005$ vs. control (at 24 h). □, control; ■, IFNγ 1 U/ml; ▤, IFNγ 10 U/ml; ▦, IFNγ 100 U/ml.

with cycloheximide displayed the typical morphological features of apoptosis, including nuclear condensation and fragmentation, with cell shrinkage. These cells were also TUNEL positive. IFNγ (100–1,000 U/ml) did not induce apoptosis in immature crypt cells, despite prolonged incubation (72 h). No significant increase of apoptotic cells was measured, even with high-dose IFNγ (3.0% apoptosis at 72 h with 1,000 U/ml IFNγ). Furthermore, assessment of cellular viability by trypan blue exclusion excluded cell death via necrosis after IFNγ treatment.

MHC class II expression

Under basal conditions (control), IEC-6 cells were MHC II antigen negative. After 24 h of incubation with IFNγ (100 U/ml), the percentage of IEC-6 cells expressing class II antigen was significantly higher ($P < 0.05$) compared to controls (Fig. 4). At 48 h of culture, even lower concentrations of IFNγ (10 U/ml) significantly enhanced IEC-6 cell expression of class II molecules ($P < 0.05$). After 72 h, IFNγ increased the number of class II-positive cells at all concentrations tested ($P <$ 0.05 to $P < 0.005$). Kinetic studies showed that 2 to 3 days was optimal for induction of class II molecules. A plateau effect was observed at a concentration of 100 U/ml IFNγ (33.2% ± 4.2, $P < 0.005$). The enhanced class II expression was completely inhibited by simultaneous addition of anti-IFNγ antibodies.

Up-regulation of class II antigen expression by IFNγ was modulated by TNFα. TNFα alone (72 h), at concentrations ranging from 0.1 to 100 ng/ml, failed to induce class II expression. However, compared to the effect of IFNγ (100 U/ml) alone, costimulation with TNFα resulted in a significant, dose-dependent enhancement of the percentage of class II-positive IEC-6 cells, concomitant with a significant gain in the fluorescence intensity (Table 1). Both parameters reached their maximum after 48 h of incubation. Figure 5 shows the relative enhancement in class II-positive cells, observed in a representative experiment, 72 h after the addition of TNFα and IFNγ (100 U/ml). Similar results were obtained in five additional experiments. At higher concentrations of IFNγ (200–800 U/ml), TNFα (10 ng/ml) did not further enhance class II antigen expression at 72 h (data not shown). This up-regulatory effect of TNFα on class II expression induced by IFNγ was confirmed by specific inhibition with anti-TNFα antibodies. As shown in Figure 6, neutralizing anti-TNFα antibodies (50 U/ml) inhibited the costimulatory effect of TNFα (10 ng/ml for 72 h) to levels similar to those induced by IFNγ (100 U/ml) alone. In contrast, neutralizing anti-IFNγ antibodies (200 U/ml) inhibited the effect induced by both cytokines together (Fig. 6).

Simultaneous measurement of cell surface class II expression and cell cycle status showed that the cells expressing MHC class II were proportionately distributed according to their number in each cell cycle phase. Addition of IFNγ (100 U/ml) or TNFα (10 ng/ml) or both changed the cell cycle distribution as well as the expression of MHC II antigen (as shown above). However, the redistribution of class II-positive cells in the different phases of the cell cycle was always proportional and did not differ under the experimental conditions. MHC class II antigen expression by IEC-6 cells was always independent of a particular cell cycle phase.

DISCUSSION

The close proximity of intestinal crypt epithelial cells and the gut-associated lymphoid tissue suggests that important interactions between these two systems are likely mediated by intercellular cross-talk. An increased number and activation of intestinal lymphocytes characterizes immune-mediated bowel pathologies, including chronic inflammatory bowel diseases,

TABLE 1. Kinetic analysis of class II MHC surface molecule expression by IEC-6 cells in response to IFNγ and TNFα[1]

Cytokines	Percentage of cells expressing class II/ median of logarithmic fluorescence intensity over background 24 h	48 h	72 h
Control	0.2 ± 2.3/3.2 ± 1.1	0.9 ± 1.8/3.9 ± 1.5	1.1 ± 3.1/5.1 ± 0.9
IFNγ 100 U/ml	12.1 ± 1.0/7.9 ± 2.9	21.3 ± 4.9/11.3 ± 2.1	33.2 ± 4.2/16.0 ± 2.8
IFNγ 100 U/ml + TNFα 0.1 ng/ml	18.4 ± 2.2/8.5 ± 2.6	45.1 ± 2.7*/17.1 ± 1.7*	52.1 ± 3.2*/16.1 ± 1.3
IFNγ 100 U/ml + TNFα 1 ng/ml	26.5 ± 2.8*/11.2 ± 1.3	55.4 ± 4.4**/24.7 ± 2.7**	56.2 ± 2.3*/17.3 ± 2.1
IFNγ 100 U/ml + TNFα 10 ng/ml	31.0 ± 3.7**/15.9 ± 1.8*	70.4 ± 3.6**/24.2 ± 2.3**	60.4 ± 6.6**/22.8 ± 3.1**

[1]Cytokines were added at 0 h (after 24 h without FCS) at the concentration specified. Cells were stained with FITC-conjugatd MRC 0X6 mouse monoclonal antibody (I-A class II specific) or an isotypic control antibody (FITC-conjugated mouse IgG_1), and analyzed by flow cytometry. Data were obtained after subtraction of the background staining values using the isotypic control FITC-conjugated mouse IgG_1, as described in Materials and Methods. These data are the mean ± standard deviation pooled from at least five separate experiments.
Statistical significance: $*P < 0.05$ and $**P < 0.005$, vs. IFNγ alone, at the same time point.

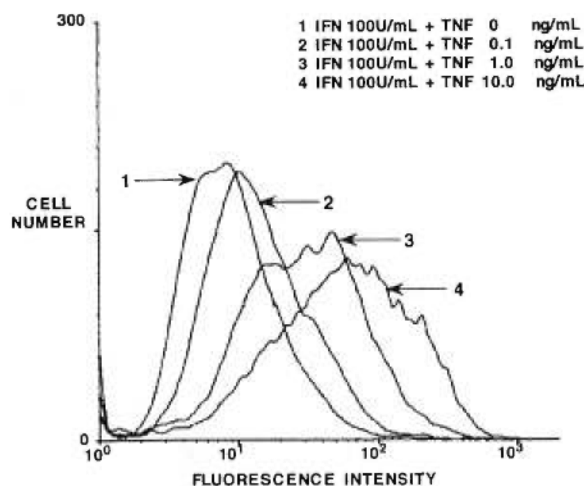

Fig. 5. Effect of TNFα on IFNγ-induced expression of class II antigens by IEC-6 cells. IEC-6 cells were cultured with TNFα (0–10 ng/ml) in combination with IFNγ (100 IU/ml) for 72 h. MHC class II expression was determined by flow cytometry. Curves represent cell number (Y-axis) vs. logarithm of fluorescence intensity (X-axis) of IEC-6 cells stained with FITC-conjugated I-A-specific monoclonal antibody. The curves were overlaid and smoothed together. The results of one representative experiment are shown.

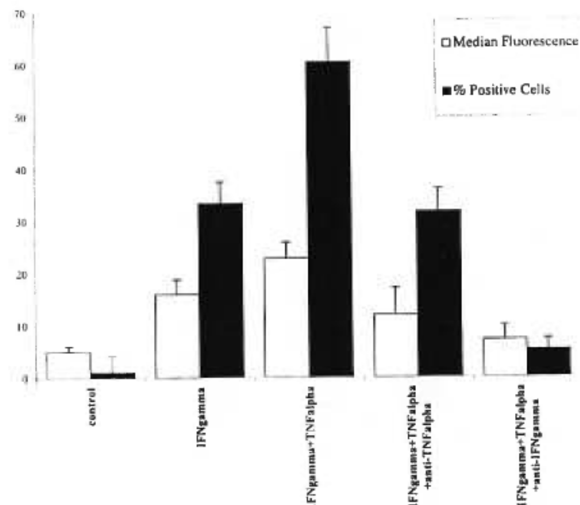

Fig. 6. Effect of blocking antibodies on TNFα and IFNγ-induced class II antigen expression by IEC-6 cells. Cells were cultured with IFNγ (100 IU/ml) plus TNFα (10 ng/ml) with or without anti-TNFα (50 U/ml) or anti-IFNγ (200 U/ml) for 72 h. Expression of MHC class II was measured by flow cytometry with FITC-labeled I-A-specific monoclonal antibody. Results shown are the mean percentage of positive cells and median fluorescence for five independent experiments (all in triplicate).

celiac disease, cow's milk allergy, or graft-vs.-host disease (GVHD; Ferguson, 1977; Phillips et al., 1979; Mowat and Ferguson, 1981). One established mechanism mediating this lymphocyte-enterocyte communication is via the local production of cytokines. Lamina propria T cells as well as intraepithelial lymphocytes (IEL) serve as a source of several cytokines including IFNγ (Ebert, 1990; MacDonald et al., 1990). Spontaneous IFNγ release by mucosal lymphocytes in Crohn's disease is increased compared to that from peripheral blood cells (Fais et al., 1991). Epithelial cells have been shown to possess receptors for several cytokines, such as IFNγ, TNFα, and transforming growth factor beta (TGFβ; Ucer et al., 1985).

Unlike previous reports using mature enterocytes or cancer cell lines, this study focused on the effects of IFNγ on nontransformed, immature rat jejunal crypt cells. We observed a marked antiproliferative effect of IFNγ on IEC-6 cells, in agreement with previous observations using ileal (Cerf-Bensussan et al., 1984) and carcinoma-derived colonic cell lines (Deem et al., 1991). Even at concentrations as low as 0.01 U/ml, IFNγ significantly inhibited IEC-6 cell proliferation. Cell cycle analysis revealed that IFNγ treatment completely blocked the exit from the quiescent G_0/G_1 into the active cell phase. TNFα can act both as a growth-inducing or inhibiting factor (Sugarman et al., 1985). We previously found that TNFα is a potent inducer of IEC-6 cell growth, with a rapid entry of IEC-6 cells into the S and G_2 + M phases, in keeping with Kaiser and Polk (1997). However, our data herein showed that when IFNγ and TNFα were administered together, the growth inhibitory effect of IFNγ predominated.

It has been suggested that the expression of class II MHC molecules may be relevant to growth regulation (Srour et al., 1992). Supernatants of αβ and wild-type IEL but not γδ T cells induced class II MHC gene expression in IEC-6 cells (Kohyama et al., 1997). This effect was mediated by IFNγ, in keeping with our re-

sults. Using cells cultured in the presence of IFNγ with or without TNFα, we did not observe that the expression of class II antigens was related to any particular phase of the IEC-6 cell cycle. However, it is difficult to conclude whether the expression of class II molecules can distinguish between cells in a true G_0 state and those in a prolonged G_1. Studies in hematopoietic cells suggested (Halper et al., 1978) that the differentiation state of the cell could determine its susceptibility to the induction of class II antigen by IFNγ and TNFα. Patterns of class II antigen expression in experimental models of GVHD (Bland and Whiting, 1992) and Nippostrongylus enteritis (Masson and Perdue, 1990) suggest that there is no clear relationship between cellular maturity and class II expression by intestinal epithelial cells. We speculate that in situ differences in local factors, such as IFNγ or TNFα, could potentially regulate class II expression in various zones of the crypt-villus axis.

IFNγ administration was shown to induce intestinal epithelial class II expression in vitro and in vivo (Kvale et al., 1988; Zhang and Michael, 1990). De novo expression of class II antigens by immature epithelial crypt cells has also been observed in several pathological situations in which the activation of mucosal lymphocytes was involved (Mason et al., 1981; Arnaud-Battandier et al., 1986; Bland and Whiting, 1992). In agreement with these studies, our results confirm that recombinant IFNγ induces de novo class II expression by IEC-6 cells. This effect was related to concentration and duration of IFNγ exposure. IFNγ-induced class II expression could represent a short-term immunoregulatory event, absent in unstimulated intestinal crypt cells. TNFα, which by itself did not significantly affect expression of class II antigen on IEC-6 cells, markedly enhanced the effect of IFNγ. In other experimental models, TNFα has been shown to enhance or inhibit IFNγ-induced class II expression (Melhus et al., 1991; Watanabe and Jacob, 1991), indicating that the effect of TNFα on class II MHC genes differs considerably between different cell types. Increased amounts of TNFα have been described in tissue from inflammatory bowel disease (Maeda et al., 1991; Braegger et al., 1992) and GVHD (Piguet et al., 1987), situations where intestinal overexpression of class II antigen is also observed. The potential risk of aberrant class II molecule expression may include enhanced self-peptide presentation, as well as altered activation signals for T lymphocytes. This could potentially lead to autoimmunity or the abrogation of oral tolerance (Sanderson et al., 1993).

IFNγ can be cytotoxic for epithelial cells and can induce a breakdown of enterocyte barrier function (Madara and Stafford, 1989; Planchon et al., 1994). Recently, it was suggested that the mucosal damage in celiac disease is predominantly mediated by IFNγ (Przemioslo et al., 1995). We did not detect any cytotoxicity or apoptosis induction by IFNγ on IEC-6 cells, even at high doses. In contrast, HT-29 cells were reported to undergo apoptosis after incubation with IFNγ (Abreu-Martin et al., 1995). This modest effect was markedly enhanced by the addition of TNFα. However, the tumoral origin of HT-29 cells, as well as the different pattern of Bcl-2-family expression in colonocytes compared to cells of the small bowel, may account for these divergent results (Moss et al., 1995). Alternatively, the varying susceptibility to IFNγ-induced apoptosis may be related to the different degree of cell maturation between HT-29 and IEC-6 cells.

The work presented herein underscores the close potential interactions between enterocytes and the adjacent mucosal immune cells mediated via cytokines. Our results support the concept (Braegger et al., 1992; Cerf-Bensussan et al., 1990) that two kinds of villus atrophy are associated with intestinal immune cell activation. In the first, where increased class II expression and decreased crypt cell mitosis are observed, as in GVHD and acute graft rejection, IFNγ production may predominate. In the second type of villus atrophy, associated with increased class II expression, crypt hyperplasia and increased mitosis, as in celiac disease or inflammatory bowel disease, TNFα may predominate. Further in vivo studies are required in order to extrapolate from our in vitro results. Since the cytokine profiles of rat and human cells may differ, direct analysis of human cells will be of interest.

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Tyrosine Kinase and MAPK Inhibition of TNF-α- and EGF-Stimulated IEC-6 Cell Growth

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The role of TNF-α in modulating intestinal crypt cell growth was examined, in comparison with EGF. Both significantly increased IEC-6 cell proliferation. Neither EGF nor TNF-α overcame the inhibitory effect on growth exerted by the tyrosine kinase inhibitor genistein. Immunoblots with phosphotyrosine antibodies showed increased tyrosine phosphorylation of IEC-6 cell proteins in response to EGF and TNF-α stimulation. TNF-α increased ERK1 and ERK2 MAPK phosphorylation. A MAPK assay confirmed the increased activity upon TNF-α stimulation. Selective inhibition of MAPK activation by PD98059 resulted in a dose dependent inhibition of TNF-α or EGF-induced IEC-6 cell growth. These findings suggest a role for TNF-α in the regulation of intestinal epithelial cell growth and that the mitogenic effect of TNF-α requires protein tyrosine phosphorylation and MAPK activation. © 1998 Academic Press

Physiologic intestinal crypt cell growth and differentiation are regulated by various growth factors and hormones, but the mechanisms involved remain largely unclear (1,2). A variety of immune-mediated intestinal inflammatory disorders, including gluten sensitive enteropathy, Crohn's disease and ulcerative colitis, are characterized by abnormal cellular proliferation and differentiation (3). Villous atrophy, crypt hyperplasia, and immature intestinal epithelial cells are common features of these diseases, resulting in diarrhea and malabsorption (1-3). Abnormal levels of cytokines and growth factors have been reported at the sites of inflammation in these pathologic conditions (4). It is therefore postulated that these mediators play a key role in the pathogenesis of the morphologic abnormalities as well as the enterocyte dysfunction observed (5).

IEC-6 cells, a non-transformed rat jejunum crypt cell line, have been widely employed in studies addressing the effect of various growth factors and cytokines on intestinal crypt cell growth and maturation (6,7). Epidermal growth factor (EGF) and transforming growth factor α (TGF-α) have been shown to stimulate IEC-6 cell growth, and to increase the phosphorylation on tyrosine residues of intracellular proteins (8,9,10).

TNF-α, a pleiotropic cytokine secreted primarily by activated monocytes, is a central mediator of diverse inflammatory processes (11). Increased amounts have been described in various inflammatory states, including Crohn's disease, ulcerative colitis and cystic fibrosis (12,13). TNF-α induces cell growth arrest or apoptosis in some cells, yet acts as a growth factor for others (14). The signalling pathways by which TNF exerts its actions remain unclear. Neither of the two TNF receptors contain intrinsic protein kinase activity. A growing number of adaptor proteins have been found to be recruited to the TNF receptors upon stimulation (15-17). These proteins elicit the liberation and/or the formation of second messengers such as ceramide, arachidonic acid metabolites, free radicals and intracellular Ca^{2+}. They also phosphorylate several proteins kinases such as PKA, PKC, MAPK, SAPK and p38 (18). The present study was conducted in order to examine the signal transduction pathways involved in TNF-α mediated IEC-6 growth. We examined the effect of tyrosine kinase inhibition, and the tyrosine phosphorylation levels of proteins in IEC-6 cells following their stimulation with TNF-α, in comparison with EGF. In addition, the role of MAPK in TNF-α induced IEC-6 cell growth was assessed.

MATERIAL AND METHODS

Cell culture and materials. IEC-6 cells (American Type Culture Collection, Rockville, MD) were initially grown at 37° C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM), containing 1% sodium pyruvate and 1% penicillin, supplemented with 0.1 U/ml insulin and 10% decomplemented fetal bovine serum (FBS, Gibco, Grand Island, NY). Cells between passages 16-20 were seeded (3 × 10^5 cells/well) into 24 well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in DMEM supplemented with 5% FBS. After reaching sub-

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confluence (48 hrs), cells were washed with phosphate buffered saline (PBS) and cultured with FBS-free medium for 24 hours.

The factors used in this study were: hTNF-α (Genzyme, Cambridge, MA) and EGF (Collaborative Research Incorporated, Bedford, MA) at concentrations ranging from 0.1 to 500 ng/ml. The inhibitors used were genistein and PD98059 (Calbiochem, San Diego, CA). At the end of the culture period, cell viability was verified microscopically, using trypan blue exclusion. Antibodies used were: monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, Lake Placid, NY), anti-phosphoMAPK (New England Biolabs, Beverly, MA), mouse anti-MAPK antibody (clone ZO33, Zymed Laboratories, San Francisco, CA), and anti-mouse IgG, conjugated to alkaline phosphatase (Bio-Rad, Richmond, CA).

Analysis of IEC-6 cell proliferation. The effect of the growth modulatory factors, of the inhibitors, and of the two combined were determined by incubating IEC-6 cells and then measuring their thymidine incorporation. Inhibitors were added 1 hour before the factors. After 18 hours of culture, ^{3}[H]-thymidine (1μCi, specific activity 20 Ci/mmol; DuPont, Markham, Ont) was added to each well. Cells were incubated for 2 hours, washed with PBS, fixed with ethanol/acetic acid (3:1) for 15 min, washed twice with PBS and solubilized with NaOH 0.2N-SDS 0.3%. In some experiments, cells were washed with PBS and then harvested with a rubber policeman in phosphate buffer (0.05 M sodium phosphate, 2 M NaCl, 0.04% sodium azide, and 2 mM EDTA, pH 7.4). Cells were sonicated and the incorporation of ^{3}[H]-thymidine in DNA was measured in homogenates (cpm/mg DNA). Results of wells treated with the factors and/or inhibitors were expressed as a percentage of thymidine incorporation relative to those IEC-6 cells maintained in medium alone (controls). Results were comparable with the two methods, with a variation of less than 10%.

Immunoblotting. IEC-6 cells seeded (1.5 × 10^6) in 25 cm^2 flasks were stimulated with TNF-α or EGF, with or without inhibitors, as described above. Lysates were prepared by washing cells with ice cold HBSS and harvesting into 0.7 ml of ice cold lysis buffer [50 mM TRIS, 150 mM NaCl, 10 mM EDTA, 1% Triton], 2mM sodium orthovanadate, 5mM sodium pyrophosphate and a mixture of protease inhibitors (Complete, Boehringer, Laval, Quebec). Lysates were clarified by a quick spin in an Eppendorf Microfuge and protein determination was performed using a modified micromethod (19).

Western blots were performed as described previously (20). Lysate samples containing equivalent protein content were mixed with 2× sample buffer (4% (w/v) SDS, 20% (v/w) glycerol, 100 mM Tris, pH 6.8, 0.5% Bromphenol Blue, 200 mM DTT) and boiled for 5 minutes. Proteins were resolved on 8%, as well as 10% polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad), and probed with the appropriate antibody. Western blots were developed as follows: initially, blocking was performed with 5% (w/v) BSA in Tris-buffered saline/Tween -20 (TBST: 20mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20), then incubated for 2 hours with the appropriate primary antibody dissolved in TBST (anti-phosphotyrosine and anti-phosphoMAPK at 1:1000 or anti-mitogen activated protein kinase (MAPK) at 1:4000), followed by incubation with the alkaline phosphatase-conjugated anti-mouse IgG (1:3000) for 2 hours. Alternatively, a peroxydase conjugated antibody (1:7000) was added and the bands were read by chemiluminescence.

MAPK assay. MAPK activity was determined in a 25 μg aliquot of cell protein using a kit (Upstate Biotechnology, Lake Placid, NY) which measures incorporation of ^{32}[P]-ATP in myelin basic protein (MBP) in the presence of PKA, PKC and calmodulin kinase inhibitors. Activity was assessed by blotting 25μl of the reaction on phosphocellulose paper and counting in a liquid scintillation counter. Specificity of the measurement was confirmed by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) analysis of radioactivity incorporation in MBP after PAGE.

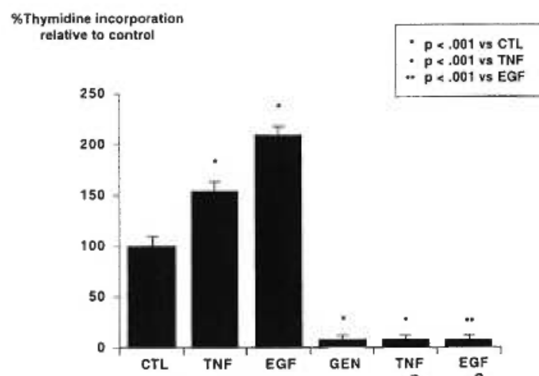

FIG. 1. Effect of EGF, TNF-α, and of the tyrosine kinase inhibitor genistein (GEN) on IEC-6 cell proliferation, measured by ^{3}[H]-thymidine incorporation. IEC-6 cells were treated with 50 ng/ml, 1 ng/ml, and 50 mg/ml of EGF, TNF-α, and genistein respectively, as indicated in the Material and Methods section.

RESULTS

Cytokine modulated IEC-6 cell proliferation. The addition of either EGF or TNF-α to the medium resulted in significantly ($p < 0.001$) increased ^{3}[H]-thymidine uptake by IEC-6 cells (Fig. 1). A significant increase was observed with incremental concentrations of TNF between 0.1 and 500 ng/ml. The dose used in experiments with inhibitors (1-10 ng/ml) represented 60% of the maximal stimulatory effect of TNF-α.

Effect of tyrosine kinase inhibition. The incubation of cells in the presence of genistein alone significantly decreased the spontaneous growth of IEC-6 cells, when compared with control wells ($p < 0.001$), as shown in Fig. 1. Cell viability, as assessed by trypan blue exclusion, was nevertheless uniformly greater than 94% throughout these experiments. Neither EGF nor TNF-α, at the doses used, was able to overcome the inhibitory effect of genistein on IEC-6 cell growth (Fig. 1).

Tyrosine phosphorylation in response to EGF and TNF-α. IEC-6 cells were incubated with 50 ng/mL EGF or 1 to 10 ng/ml TNF-α for 1 to 45 minutes. Immunoblotting revealed that several proteins were rapidly phosphorylated by EGF, with approximate molecular masses of 42, 44, 80, 150, and 175 kDa (Fig. 2A, lane 2). TNF-α induced phosphorylation of proteins with approximate molecular masses of 42, 44, 55, 85, and 110 kDa (Fig. 3A, lane 2). The EGF induced phosphorylation of the proteins corresponding to the 80 and 150 kDa bands was transient, starting at 1 min (not shown). The most prominent and sustained phosphorylation induced by TNF-α was observed for the protein corresponding to the 55 kDa band. Although EGF and TNF-α both induced phosphorylation of proteins corre-

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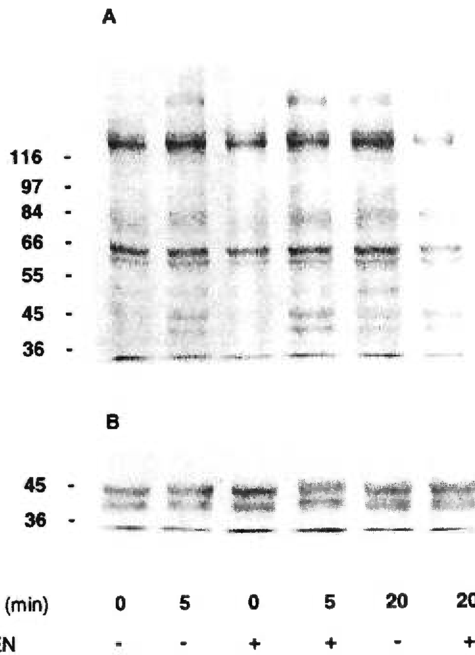

FIG. 2. Effect of EGF and genistein (GEN) on tyrosine phosphorylation in IEC-6 cells. IEC-6 cells were incubated with DMEM alone for 5 or 20 minutes (lanes 1, 4), with 50 ng/ml EGF for 5 or 20 minutes (lanes 2, 5), or with 50 ng/ml genistein and EGF for 5 or 20 minutes (lanes 3, 6). (**A**) Immunoblot illustrating that EGF increased the phosphorylation on tyrosine residues of proteins of approximate molecular weights of 175, 150, 80, 44, and 42 kDa, respectively. Phosphorylation of these proteins was inhibited by genistein. (**B**) Immunoblot performed with anti-MAPK. The two bands in lane 2 of immunoblot B appear to be located at a higher molecular mass than the corresponding bands in lane 1 (control), likely indicating a shift or activation (by phosphorylation) of MAPK in response to EGF.

sponding to the 42 kDa and 44 kDa bands, the phosphorylation induced by EGF was more prominent, and appeared sooner. EGF greatly increased phosphorylation levels of these proteins with peak at 3 to 5 min, afterwhich the phosphorylation decreased progressively but remained above basal levels to 45 min. The phosphorylation levels induced by TNF-α increased only slightly at 5 min, were higher by 10 min and returned to basal levels by 30 min.

When immunoblots were performed using the anti-MAPK antibody, the 42 and 44 kDa isoforms aligned precisely with p42 and p44 (Fig 2B, 3B). These bands were observed at slightly higher molecular masses than those of the control cells, implying their phosphorylation subsequent to EGF or TNF-α stimulation.

Effect of tyrosine phosphorylation inhibition. Preincubating IEC-6 cells with genistein (50 ng/ml for 1 h) caused a decrease in the EGF and TNF-α induced tyrosine phosphorylation of most proteins, especially that of the 42 and the 44 kDa bands (Figs 2A, 3A). Inhibition was incomplete at 50 ng/ml, but higher doses of genistein were cytotoxic.

Effect of MAPK inhibition. Pre-incubation of cells with the selective MAPK kinase (MEK) inhibitor PD98059 decreased spontaneous IEC-6 cell proliferation by 22 and 30% at 20 and 40μM, respectively. PD98059 at 20 μM decreased TNF-α and EGF-induced proliferation by 38 and 41%, respectively. At 40 μM, TNF-α and EGF-induced IEC-6 cell proliferation was decreased by 63 and 67%, respectively (Figure 4). Pre-incubation of IEC-6 cells with 100 μM PD98059 partially inhibited basal (Figure 5, lane 2) as well as TNF-α -induced (Figure 5, lane 4) MAPK phosphorylation. The MAPK kinase assay showed that radioactivity incorporation in MBP was increased by 24% by TNF-α, while EGF induced MAPK activity by 1.5 to 1.8 fold (Table 1). Treatment with PD98059 reduced the TNF-α induced activity by approximatively 50% (11 vs 24% increase over basal value).

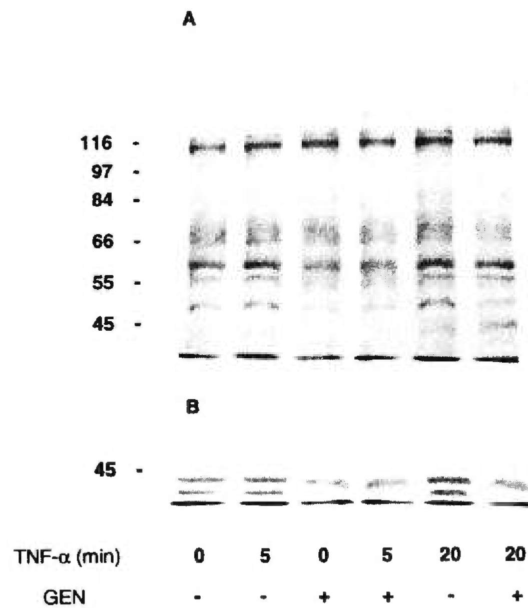

FIG. 3. Effect of TNF-α and genistein (GEN) on tyrosine phosphorylation in IEC-6 cells. IEC-6 cells were incubated with DMEM alone for 5 or 20 minutes (lanes 1, 4), with 1 ng/ml TNF-α for 5 or 20 minutes (lanes 2, 5), or with 50 ng/ml genistein and TNF-α for 5 or 20 minutes (lanes 3, 6). (**A**) Immunoblot illustrates that TNF-α increased the phosphorylation on tyrosine residues of proteins of approximate molecular mass of 110, 85, 55, and 44 kDa, respectively. A fainter band was also noted at 42 kDa. The bands all appear more intense at later time points (lane 5 vs lane 2; 20 vs 5 min stimulation with TNF-α). Preincubation with genistein partially inhibited phosphorylation of these proteins. (**B**) Immunoblot performed with anti-MAPK. A shift upwards in molecular mass is less evident compared to EGF stimulated cells (lanes 2 and 5).

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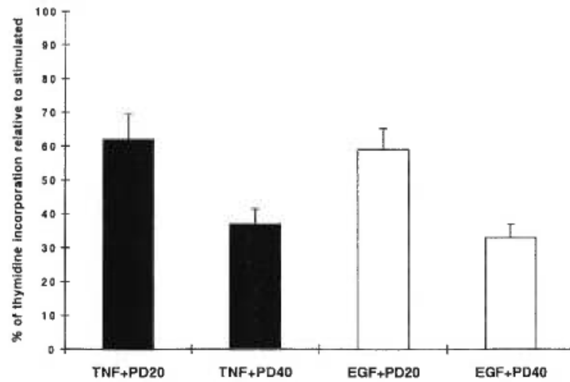

FIG. 4. Incubation of IEC-6 cells with the selective MAPK kinase inhibitor PD98059 decreased TNF and EGF-induced proliferation ($p \leq 0.01$). Results are expressed as percent of cytokine treated cells without an inhibitor.

DISCUSSION

Our results demonstrate that TNF-α increased IEC-6 cell proliferation over a broad range of concentrations. This implies that TNF-α may act as a mitogen for intestinal crypt cells, resulting in the increased epithelial cell turnover encountered in inflammatory states. Genistein abolished spontaneous as well as EGF or TNF-α induced proliferation, suggesting that tyrosine kinase activation is essential to IEC-6 cell growth. TNF-α elicits rapid tyrosine phosphorylation of several proteins (21). That EGF and TNF-α promote the stimulation of tyrosine kinase was shown by the increased phosphorylation on tyrosine of several IEC-6 cell proteins. It is well established that the phosphorylation on tyrosine residues of certain proteins is closely linked to the initiation of cell proliferation (22). Protein tyrosine kinases also are involved in the regulation of intestinal crypt cell growth when stimulated by gastrin (23).

TNF	-	-	+	+	+	-
EGF	-	-	-	-	-	+
PD98059	-	+	-	+	-	-

FIG. 5. Effect of TNF, EGF and PD98059 on MAPK tyrosine phosphorylation. Western blotting was performed with a phospho-specific antibody that detects phosphorylated tyrosine 204 of p42 and p44 MAPK. TNF (10 ng/ml, 10 min) increased MAPK phosphorylation (lane 3). At 30 min, MAPK phosphorylation was returned to basal levels (lane 5 vs lane 1). PD98059 decreased basal (lane 2) as well as TNF-induced (lane 4) MAPK phosphorylation. EGF (50 ng/ml, 10 min) elicited a stronger MAPK phosphorylation (lane 6).

TABLE 1

MAPK Activity in Stimulated IEC-6 Cells

TNF (1 ng/ml, 10 min)	124 ± 3%	(n = 4)
TNF + PD98059	111 ± 2%	(n = 3)
EGF (50 ng/ml, 3 min)	182 ± 4%	(n = 3)
EGF (50 ng/ml, 15 min)	151 ± 4%	(n = 3)

TNF treatment increased MAPK activity, which was partially blocked by PD98059 (100 μM, 1 h). Activity is given as % of non stimulated.

Furthermore, altered levels of protein tyrosine kinases have been detected in biopsy specimens obtained from patients with ulcerative colitis (24), an immune-mediated bowel disorder with enhanced crypt cell turnover.

It is of interest that MAPK were phosphorylated in IEC-6 cells in response to both EGF or TNF-α. MAPK are a family of serine-threonine kinases which constitute an important intermediate step in the network of cellular signal transduction pathways, spanning from protein tyrosine kinase receptors to downstream transcriptional events (25). The phosphorylation of MAPK on tyrosine and threonine activates the enzyme, which in turn phosphorylates several substrates involved in the regulation of cell growth, including c-fos, elk1, p90rsk, and others (18). Activation of MAPK by TNF-α was reported for several cell types such as fibroblasts, in which it stimulated proliferation (26). While EGF activation of MAPK in IEC-6 cells has been recently reported (10), we are the first to demonstrate MAPK activation in TNF-α stimulated IEC-6 cells. Activation of two independent signalling pathways have been defined upon binding of TNF to TNF-Rp55. The first one involves neutral sphingomyelinase, ceramide-activated protein kinase and Raf-1 kinase, while the other leads to NF-KB activation through phosphatidylcholine-specific phospholipase C, PKC and acid sphingomyelinase (27). MAPK activation was located in the first pathway in which Raf is believed to activate MAPK through MEK (28). However, MAPK activation by TNF was reported to be independent of raf-1 in some cells (29).

The molecular mechanisms that make a cell resistant or sensitive to TNF-induced apoptosis have not been clarified. It has recently been proposed that TNF stimulates simultaneous pathways: some that convey programmed cell death and others that protect against apoptosis (30). Initial studies pointed to a role of MAPK in cell survival, while SAPK and p38 kinase activation was associated with apoptosis (31,32). However no role for SAPK in apoptosis was found in other studies. (15, 33). Furthermore, MAPK activation is not sufficient to protect against apoptosis in certain cells (34). There is now evidence that TNF-α can activate several MAPK pathways (ERKs, SAPK and p38)(35). One recently identified protective pathway elicited by TNF is NFκB activation (30,36). In addition to the MAPKs ERK 1

and ERK2, we observed that TNF-α-induced transient phosphorylation of SAPK and p38 kinase in IEC-6 cells (data not shown). The role of these kinases in TNF-induced proliferation vs programmed cell death is currently under investigation.

In summary, our results show that TNF-α stimulates IEC-6 intestinal crypt cell proliferation and that this mitogenic effect is in large part mediated by MAPK activation. Establishing that TNF-α stimulates the phosphorylation of tyrosine residues on MAPK and other regulatory proteins will increase our understanding of the signal transduction mechanisms of TNF-α modulation of intestinal epithelial cell growth and function.

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TNFα-INDUCED IEC-6 CELL APOPTOSIS REQUIRES ACTIVATION OF ICE-CASPASES WHEREAS COMPLETE INHIBITION OF THE CASPASE-CASCADE LED TO NECROTIC CELL DEATH

Running title: TNFα signaling in enterocyte apoptosis

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ABSTRACT

Tumor necrosis factor (TNF)α plays a pathogenetic key role in inflammatory bowel diseases. In this study we analyzed the mechanisms of TNFα induced IEC-6 cell apoptosis. TNFα alone and more potently in the presence of IFNγ, induced a high degree of IEC-6 cell apoptosis. This effect was more than 100-fold stronger if both TNF-R were stimulated, compared to the p55-TNF-R alone, indicating an important enhancing effect of the p75-TNF-R. TNFα induced apoptosis required activation of ICE-caspases and was completely abolished by its inhibitor zVAD-fmk, whereas specific inhibition of caspase-3 with zDEVD-fmk had no effect. Western blot analyses confirmed that caspase-3 was not activated in response to TNFα. Complete inhibition of the caspase-cascade with high zVAD-fmk doses (50 µM) switched TNFα induced apoptosis to necrosis. Our data revealed that TNFα triggers enterocyte cell death via two different pathways, resulting in apoptosis or necrosis depending on the activation of specific caspases.

INTRODUCTION

Tumor necrosis factor (TNF)α, a pleiotrophic, proinflammatory cytokine, plays a crucial role in the pathogenesis of various inflammatory states, including idiopathic chronic inflammatory bowel diseases (IBD, 1,2). TNFα binds to two distinct membrane receptors, the p55 and p75 TNF receptors (TNF-R), both of which are expressed by intestinal epithelial cells (3). In addition to their involvement in immune regulation and inflammation (4), the TNF-R have been implicated in the induction of apoptosis, activation of nuclear factor kappa-B and regulation of cell proliferation via mitogen-activated protein kinases or N-terminal c-Jun kinase (5-7).

The p55 TNF-R is primarily responsible for TNF-induced apoptosis of target cells (8,9). Analysis of the molecular mechanisms has revealed that the cytoplasmatic death-domain (DD) of the p55 TNF-R is necessary to trigger the apoptotic response upon stimulation (10). The interaction of DD with intracellular adapter molecules, such as TRADD, FADD or others, allows the formation of a death-inducing signaling complex (DISC), resulting in rapid activation of pro-caspase-8 and subsequently the caspase-cascade, leading to apoptotic cell death in various models (10,11). In contrast to the p55, the p75 TNF-R lacks an intracellular domain that induces apoptosis. To date, the contribution of the p75 TNF-R in mediating apoptosis is controversial and its signaling mechanisms remain unclear.

The cascade of intracellular aspartate-specific cysteinyl proteases, now referred to as caspases, is critical to apoptosis (12). These intracellular caspases are present as inactive zymogens that become activated after cleavage into two subunits in response to apoptotic stimuli. Recently, a model was proposed (12) dividing caspases into initiator proteases, such as caspases-8 or -9, while others, such as caspases-3, -6 or -7, act as potent effectors of apoptosis. This latter group of caspases is responsible for the degradation of various enzymes and structural proteins involved in apoptotic cell death (12).

The aim of this study was to analyze the role and underlying signaling mechanisms of TNFα in inducing intestinal epithelial cell apoptosis. The model employed is the IEC-6 cell line, non-transformed immature intestinal epithelial cells of rat origin (13). TNFα-induced apoptosis was more than 100-fold stronger if both TNF-R were stimulated, compared to the p55 TNF-R alone, indicating an important enhancing effect of the p75 TNF-R. The apoptotic signaling required activation of ICE-caspases, but was independent of caspase-3. Complete inhibition of the caspase-cascade with zVAD-fmk switched the apoptotic response to TNFα into a necrotic form of cell death.

Material and Methods

Cell culture and reagents: IEC-6 cells (passages 17-20, ATCC, Rockville, MD) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY), 1 mM sodium pyruvate, 1% penicillin/ streptomycin, as described (14). The cell monolayers were stimulated with recombinant human (h) TNFα, which selectively stimulates the p55-TNF-R of rat cells without binding to the p75 TNF-R (15), or recombinant rat (r) TNFα, which stimulates both the p55 and p75 TNF-R (both from Genzyme, St-Louis, MO, at 0.01 to 1000 ng/ml). To control for the specificity of each cytokine source, neutralizing rabbit anti-human TNFα (Peprotech, Rocky Hill, NJ) or rabbit anti-rat TNFα (Genzyme) antibodies were employed (50 neutralizing U/ml). Further reagents used in this study were: IFNγ (0.1-100 U/ml, R+D), caspase-3 specific inhibitor zDEVD-fmk, ICE-like caspase specific inhibitor zVAD-fmk (both from Kamyia, Thousand Oaks, CA), polyclonal anti-caspase-3 antibodies (Pharmingen, Mississauga, Ont. and rat-specific from Santa Cruz, Santa Cruz, CA) which both recognize the native (32kDa) and the proteolytically active forms (17kDa), horseradish peroxidase-labelled antibodies directed against mouse or rabbit immunoglobulins (Ig)G (both from Promega, Madison, WI), or anit-goat-IgG (Biosource, Camarillo, CA), propidium iodide (PI, Sigma), and HOECHST 33342-DNA stains (Boehringer-Mannheim, Germany).

Apoptosis assays: IEC-6 cells were cultured in 24-well-plates (Falcon) at a density of 1-6x10^5 cells/ml. Cells were allowed to adhere overnight, after which the medium was changed. The cells were then treated for up to 48h with 0.1 to 1000 ng/ml hTNFα or rTNFα alone, or in the presence of IFNγ (1-100 U/ml). After harvesting (floating cells included), the cells were stained with PI (5µg/ml, Sigma, St.-Louis, MO) or the Hoechst 33342 DNA-dye (5µg/ml) on ice and immediately analyzed by flow cytometry (PI staining) as well as by fluorescence microscopy (PI+Hoechst staining). In parallel, the TUNEL-assay (Boehringer-Mannheim) was used to confirm apoptotic cell death, as previously described (14). Furthermore, the expression of phosphatidylserine on the outer plasma-membrane, characteristic of early apoptotic cells, was monitored using the Apo-Alert Annexin V kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. Apoptosis was then quantified by flow cytometry (FACScan, Becton Dickinson, Missisauga, ON). Dose-response studies showed that 50 ng/ml concentrations of rTNFα or IFNγ+hTNFα induced a submaximal rate of apoptosis Therefore, this concentration was used to perform inhibitor studies, unless otherwise indicated. To investigate the signaling pathways involved in TNFα-induced apoptosis, specific anti-caspase-3 (zDEVD-fmk) and

anti-ICE-like caspases (zVAD-fmk) peptide-inhibitors, which interact with the active site of the cleaved caspases, were used (at 0.01-500 µM) under the same experimental conditions as above. To confirm the biological effect of these caspase-inhibitors, FAS-induced apoptotic Jurkat cells served as control model (data not shown).

Immunoblotting: The activation of caspase-3 was determined by western blotting. TNFα stimulated IEC-6 cell lysates were prepared using an ice-cold lysis buffer (50mM Tris, 150mM NaCl, 10 mM EDTA, 1% Triton and a mixture of protease inhibitors (Boehringer), as previously described (7). After determination of protein concentrations, equivalent samples were resolved on 14% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). For immunodetection, the membranes were incubated overnight with either of two polyclonal anti-caspase-3-antibodies (1:1000 Pharmingen or 1:50 Santa Cruz) diluted in Tris-buffered saline/Tween-20-1% milk powder, followed by incubation with the corresponding alkaline phosphatase-conjugated antibody (anti-rabbit-IgG 1:2500, or anti-goat-IgG 1:5000). The bands were read by enhanced chemiluminescence (ECL-kit, Amersham). Both antibodies detected active caspase-3 as 17 kDa band in IEC-6 cell lysates, when the membranes were overexposed, indicating minimal caspase-3 degradation under unstimulated conditions. Apoptotic Caco-2 cells (induced by butyrate-treatment) served as positive control for caspase-3 detection.

Proliferation Assays: IEC-6 cell growth was examined by monitoring changes in DNA synthesis, as measured by the incorporation of ^{3}H-thymidine (1µCi/ml) into cellular DNA for 2h prior to the termination of cultures, as we have previously described (14).

Experimental design and statistical analysis: All apoptosis and proliferation experiments were performed in triplicate and were repeated at least ten times, western blot experiments were repeated at least four times to ascertain reproducibility. Representative experiments or mean values ± standard deviation are shown. Significance was established at 95%, and determined by the Mann-Whitney U-test.

RESULTS

Effect of TNFα on IEC-6 cell apoptosis. rTNFα potently induced IEC-6 cell apoptosis, in a dose- and time-dependent manner, as analyzed by TUNEL-assay and Annexin-V staining (Fig.1). To address the question, if TNFα-induced IEC-6 cell apoptosis was solely mediated via the p55 TNF-R, as described for other models, this receptor was selectively stimulated with hTNFα. As shown in Fig.1, only a weak apoptotic response was observed at very high hTNFα concentrations (1000 ng/ml). At least 100-fold higher TNFα doses were required to induce IEC-6 cell apoptosis when the p55 TNF-R alone was stimulated, compared to the effect of rTNFα, which stimulates both TNF-R. Co-stimulation with IFNγ, alone without apoptotic effect, as previously demonstrated (14), rendered IEC-6 cells more susceptible to TNFα-induced apoptosis (Fig.1). IFNγ enhanced the apoptotic response to hTNFα or rTNFα in a similar manner. No change in the constitutive expression of the p55 or p75 TNF-R was observed after stimulation with IFNγ (data not shown). In comparison, the apoptotic response of IEC-6 cells to isolated stimulation of the p55 TNF-R in the presence of IFNγ, was still weaker than p75 TNF-R enhanced apoptosis seen with rTNFα. IEC-6 cell apoptosis in response to rTNFα was confirmed by morphological analysis (Fig.2), as shown by nuclear condensation, fragmentation with formation of apoptotic bodies. These findings were similar to IFNγ+hTNFα induced apoptosis via the p55 TNF-R alone (Fig. 2d).

TNFα-induced apoptosis requires activation of ICE-caspases. In order to unravel the signaling mechanisms of TNFα-induced apoptosis via the p55 TNF-R alone or both TNF-R, IEC-6 cells were stimulated with hTNFα or rTNFα alone or in combination with IFNγ in the presence of the irreversible caspase-inhibitors zVAD-fmk or zDEVD-fmk. As shown in Fig.3a, the ICE-like caspase inhibitor zVAD-fmk (0.01-10 µM) potently blocked the apoptotic effect of rTNFα alone and in combination with IFNγ, as well as the combination of IFNγ+hTNFα. In contrast, the caspase-3 specific peptide-inhibitor zDEVD-fmk failed to block rTNFα and IFNγ-facilitated TNFα mediated apoptosis. Only at zDEVD-fmk concentrations of 200 µM and higher, an anti-apoptotic effect was observed. In this dose range, however, the peptide's effect is less specific and interaction with other caspases than the CPP-32 group cannot be excluded. In addition, western blot analysis revealed no proteolytic activation of caspase-3, typically seen as 17kDa band or indirectly as diminished expression of the pre-active caspase form (32kDa, Fig. 3b).

Effect of complete caspase-cascade inhibition with high zVAD-fmk doses on TNFα-induced IEC-6 cell death. Unlike the anti-apoptotic effect of zVAD-fmk at low concentrations (10 µM), a dramatically increased number of dead cells in response to

rTNFα (maximally: 63%) was observed with zVAD-fmk doses of 50 μM or higher (Fig.4). This cell death sensitizing effect was also observed after isolated stimulation of the p55 TNF-R with hTNFα, even at cytokine concentrations that failed to induce apoptosis. Morphological analysis of these cells showed no signs of apoptosis, but rather, necrotic cell death with swelling of the cell membrane and nucleus (Fig.2e). In addition, no formation of apoptotic bodies was detected in these cells. Parallel experiments with zVAD-fmk alone, however, revealed no cytotoxic effect, at all doses tested. Furthermore, control experiments with the dissolvent DMSO alone and in combination with hTNFα or rTNFα did not induce IEC-6 cell necrosis, indicating that the observed effect was due to complete inhibition of the caspase-cascade at high zVAD-fmk concentrations. No shift towards necrotic cell death was observed in response to TNFα in the presence of zDEVD-fmk, in contrast to zVAD-FMK.

The p55 TNF-R is necessary and sufficient to stimulate IEC-6 cell growth. In contrast to the apoptotic response, which was more potent after simultaneous activation of both TNF-R, stimulation of the p55 TNF-R alone was sufficient to enhanced IEC-6 cell proliferation, as demonstrated by increased DNA synthesis (^{3}H-thymidine incorporation, Fig.5). The growth promoting effect of hTNFα and rTNFα was detectable at doses as low as 0.1 ng/ml and it was maximal at 10 ng/ml (291±18% and 311±30.6%, respectively, $p<0.001$). This promitogenic TNFα effect was not significantly different, if hTNFα or rTNFα was used.

DISCUSSION

TNFα is believed to play a key role in the pathogenesis of IBD. Recent reports of the successful use of anti-TNFα antibodies in the treatment of Crohn's disease further underscored the importance of this particular cytokine in the pathogenesis of chronic idiopathic bowel disorders (16). To date, however, the understanding of the underlying molecular mechanisms as to how TNFα contributes to intestinal inflammation and to the damage of the intestinal epithelium is very limited. In this study, we clearly demonstrated that TNFα is a potent inducer of intestinal epithelial cell death.

The parallel use of recombinant hTNFα, which selectively stimulates the p55 TNF-R, and rTNFα, which stimulates both TNF-R (15), allowed us to further analyze the distinct roles and signaling mechanisms of these receptors in mediating IEC-6 cell apoptosis. Costimulation of both TNF-R was significantly more potent in inducing IEC-6 cell apoptosis, compared to the p55 TNF-R alone. In order to explain these findings ligand passing mechanisms or cross-talk between the two TNF-R were proposed, rather than independent signaling via the p75 TNF-R to induce apoptosis (17). Using Hela transfectants expressing wild-type p75 TNF-R or deletion mutants, Weiss et al. (18) suggested that distinct intracellular signaling mechanisms are required for the synergistic activity between the two TNF-R. It was proposed that the interaction of TNF receptor-associated factor (TRAF)-2 and the p75 TNF-R is relevant to the enhancement of p55 TNF-R mediated cytotoxicity by the p75 TNF-R (18). Our data largely support this concept of receptor crosstalk. To induce IEC-6 cell apoptosis, over 100-fold higher TNFα doses were required when the p55 TNF-R alone was stimulated compared to activation of both TNF-R, confirming the important enhancing effect of the p75 TNF-R. On the other hand, TNFα-induced proliferation, which was solely mediated via the p55 TNF-R, was almost similar in response to either cytokine, indicating comparable biological activities of both recombinant factors.

In an attempt to gain insight into the signaling mechanisms underlying TNFα induced apoptosis in our enterocyte cell model, we selectively blocked different caspases, known to be essential to apoptosis (12). The specific caspase-3 inhibitor zDVED-fmk failed to block TNFα-induced apoptosis. A moderate anti-apoptotic effect was only observed when high concentrations of this inhibitor were used. Non-specific inhibition of other caspases than the CPP-32 group cannot be excluded at these doses. Western blot analyses confirmed that this particular caspase was not activated in response to TNFα, even after prolonged stimulation (48h). Furthermore, in the presence of IFNγ, which markedly

enhanced TNFα-induced apoptosis, no significant activation of caspase-3 was observed. Ossina et al. (19) recently showed that the sensitizing effect of IFNγ to TNFα-induced apoptosis in HT-29 colon cancer cells, was mediated via increased gene expression of several caspases, such as caspase-3. We did not detect an increased caspase-3 expression after stimulation of IEC-6 cells with IFNγ, as analyzed by western blots (unpublished results). Furthermore, the expression of ICE-caspases was particularly enhanced by IFNγ in HT-29 cells (19). This is in accordance with our findings in IEC-6 cells that TNFα-induced apoptosis required activation of ICE-caspases and IFNγ potently enhanced this pro-apoptotic effect of TNFα. Treatment with the ICE-inhibitor zVAD-fmk completely abolished the apoptotic response to rTNFα and to a high degree IFNγ-enhanced apoptosis after stimulation with hTNFα or rTNFα. This is in keeping with the observation of Cryns et al. (20) that TNFα-induced apoptosis required the activation of ICE-caspases to cleave of the membrane-associated cytoskeletal protein α-fodrin. Recently, Garcia-Calvo et al (21) showed that zVAD-fmk is a broad-range inhibitor binding to all caspases but with varying affinity. At low doses, it selectively inhibits caspases-1, 4 and 11, but at higher concentrations it also inhibits caspases of the other groups and probably yet not identified caspases (22,23). We did not observe any differences in the apoptotic signaling in response to isolated stimulation of the p55 or both TNF-R supporting the hypothesis that the enhancing effect of the p75 TNF-R results in activation of similar signal transduction pathways as the p55 TNF-R. Surprisingly, complete inhibition of ICE-caspases, with zVAD-fmk at concentrations of 50 μM or higher markedly increased the number of dying IEC-6 cells in response to both, isolated p55 TNF-R stimulation or simultaneous activation of both TNF-R. Morphological analysis confirmed that the initially apoptotic form of cell death was switched to a necrotic form due to the inhibition of essential effectors of the apoptotic pathway. These findings expand upon the recent observations of FAS and TNF-induced dual signaling resulting in apoptosis or necrosis in the L929 cell model (24,25). Vercammen et al. (24) proposed two different death pathways originating from FAS: one rapidly inducing apoptosis, and if it is blocked, a second resulting in necrotic cell death involving mitochondrial generation of oxygen radicals. Similar conclusions might apply to the TNF-R pathways in the IEC-6 cell model. It is important to note, that even TNFα doses, which failed to induced apoptosis, potently triggered necrosis in the presence of complete caspase-inhibition. This might be of particular importance in view of potential clinical trials with specific caspase-inhibitors for GI-diseases.

In summary, TNFα is a potent inducer of enterocyte death. The underlying signaling mechanisms revealed that TNFα can activate two different cell death pathways.

The apoptotic pathway required activation of ICE-caspases, whereas the necrotic pathway occurred only after complete inhibition of the caspase-cascade. These findings indicate that caspases play a protective role against necrotic cell death in the intestinal epithelium.

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FIGURE LEGENDS

Fig.1. **TNFα-induced apoptosis in IEC-6 cells.** Selective stimulation of the p55 TNF-R with hTNFα alone only induced apoptosis at very high concentrations. However, in response to rTNFα, which stimulates the p55 and p75 TNF-R, a high degree of apoptosis was observed, in a dose-dependent manner. In addition, co-stimulation with IFNγ rendered the cells more susceptible to TNFα-induced apoptosis via the p55 TNF-R alone or both TNF-R. The mean of six independent experiments ± standard deviations are shown. * $p<0.01$.

Fig.2. **Morphological analysis of TNFα-induced IEC-6 cell apoptosis**. Staining with the HOECHST 33342 DNA-dye was utilized in order to obtain a detailed morphological analysis confirming apoptosis, by immunofluorescence microscopy. Control cells (a) displayed ovaloid nuclei with normal heterochromatin structure. In contrast, rTNFα-stimulated IEC-6 cells showed typical nuclear fragmentation with the formation of apoptotic bodies (b), or marked DNA-condensation (c), both characteristic of advanced stages of apoptosis. Similarly, IFNγ-facilitated p55 TNF-R triggered apoptotic cells are observed to be fragmented into typical apoptotic bodies (d). However, in the presence of high doses of the caspase-inhibitor zVAD-fmk (100 μM), rTNFα- stimulated cells died by necrosis (e), characterized by swelling of the nuclei and a loss of the normal chromatin structure.

Fig. 3. **IEC-6 cell p75 TNF-R mediated apoptosis requires the activation of ICE-caspases.** a) Specific inhibition of caspase-3 with zDEVD-fmk failed to abolish rTNFα (50 ng/ml)-induced IEC-6 cell apoptosis, whereas the ICE-caspase inhibitor zVAD-fmk potently reduced the apoptotic effect. However, at zVAD-fmk doses of 50 μM or higher, a marked increase in the number of dead cells in response to rTNFα was noted. Similarly, apoptosis in response to isolated stimulation of the p55 TNF-R (hTNFα, 50 ng/ml)in the presence of IFNγ (100 U/ml) was significantly suppressed by zVAD-fmk. Apoptosis was quantified by the Annexin-V-assay. The mean data of six independent experiments performed in duplicates are shown ± standard deviation. b) Western blot analysis of hTNFα or rTNFα stimulated IEC-6 cell showed no proteolytical activation of caspase-3, confirming the observation that TNFα-induced apoptosis was independent of caspase-3. The positive control consisted of butyrate-induced apoptotic Caco-2 cells.

Fig. 4. **TNFα induced necrosis after complete inhibition of the caspase-cascade.** In the presence of high doses zVAD-fmk (100 μM), isolated stimulation of the

p55 TNF-R and more potently stimulation of both TNF-R induced IEC-6 cell necrosis in a dose-dependent manner.

Fig. 5 **Stimulation of the p55 TNF-R potently induced IEC-6 cell proliferation**. Both r TNFα and hTNFα stimulated IEC-6 cell proliferation in a dose-dependent way, as determined by ^{3}H-thymidine incorporation. However, at rTNFα concentrations of 100 ng/ml, this trophic effect was less due to the simultaneous cytotoxic effect. Mean data of five independent experiments are shown. * $p<0.05$ versus control.

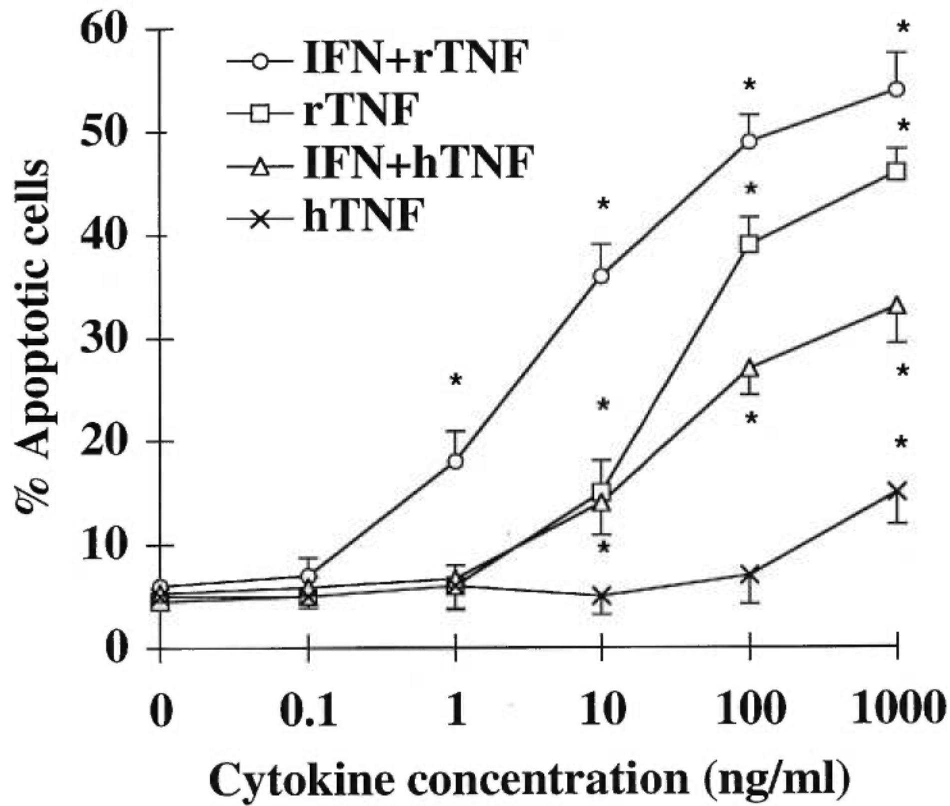


Fig.1

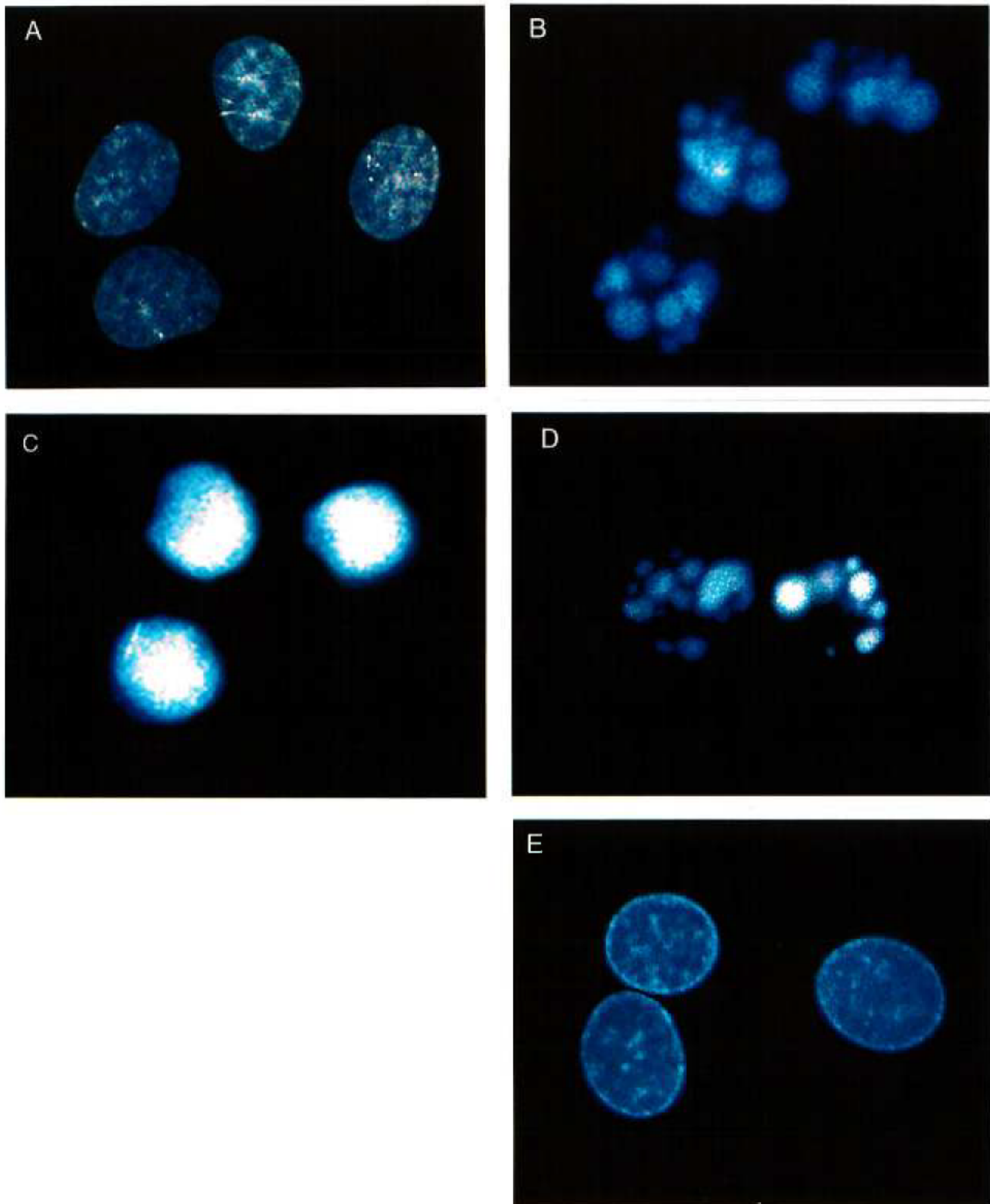
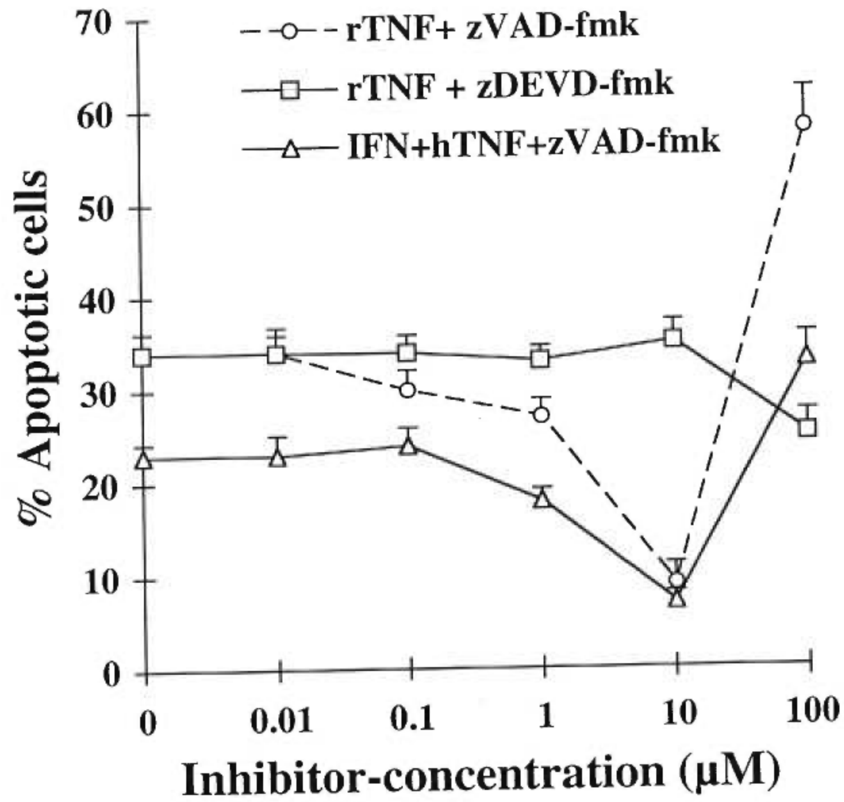


Fig.2



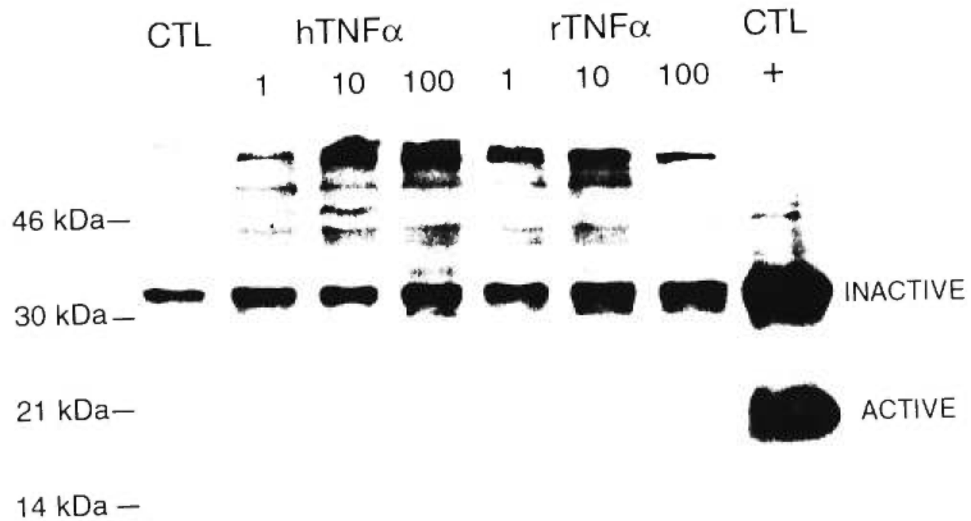


Fig.3

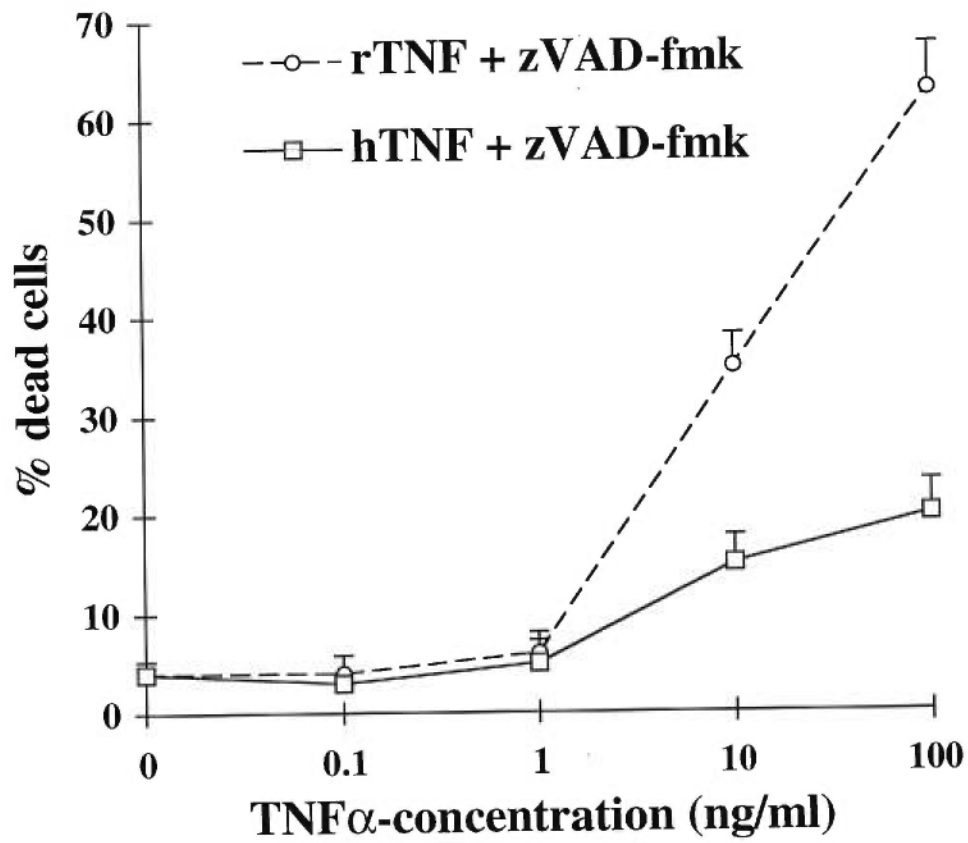


Fig.4

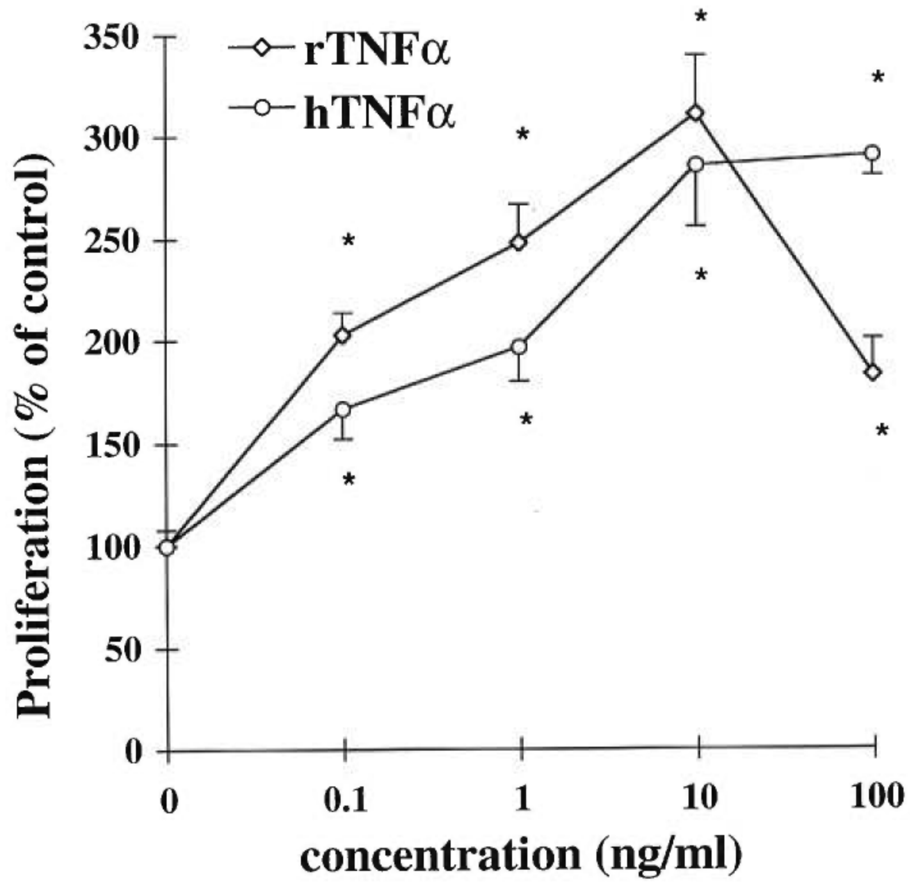


Fig.5

SUSCEPTIBILITY TO FAS-INDUCED APOPTOSIS IN HUMAN NON-TUMORAL ENTEROCYTES - THE ROLE OF COSTIMULATORY FACTORS

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Abstract

FAS-FAS ligand interaction is implicated in increased enterocyte apoptosis seen in immune-mediated bowel injury. However, scant information exists on the role of FAS in physiological enterocyte turnover. In this study, the regulation of enterocyte FAS and FAS ligand expression by cytokines and its functional role in human intestinal epithelial cell apoptosis and proliferation was analyzed using two different models: a non-transformed human intestinal epithelial cell line (HIEC) and normal colonic explant cultures. HIEC constitutively expressed FAS, as analyzed by flow cytometry. However, stimulation with agonistic anti-FAS antibody (1-500 ng/ml) did not induce HIEC apoptosis. In contrast, in the presence of TNFα and/or IFNγ, HIEC became highly susceptible to FAS-induced apoptosis. The sensitizing effect to FAS-induced apoptosis was mediated via TNFα and IFNγ–induced upregulation of FAS expression (maximally 348%). Receptor-studies revealed that the TNFα effect on FAS was mediated via the p55 TNF-receptor. In colonic organ cultures, IFNγ and TNFα also enhanced colonocyte FAS expression, resulting in a markedly increased apoptotic response to stimulation of this receptor, as shown by *in situ* TUNEL staining. No FAS ligand expression, nor its induction by cytokines was observed in HIEC or colonic explants. Proliferation studies showed that FAS is not implicated in regulating HIEC growth. These findings suggest that, despite the fact that normal human enterocytes express FAS, costimulatory factors, such as TNFα or IFNγ, abundantly secreted under inflammatory conditions, are necessary to sensitize intestinal epithelial cells to FAS-induced apoptosis via upregulating this receptor.

Introduction

Apoptosis or programmed cell death is the normal fate of mature enterocytes at the end of their lifespan. Apoptotic cells shed from the villus epithelium into the gut lumen are normally replaced by an equal number of proliferating, immature crypt cells. Under physiological conditions, apoptotic cells are restricted to the villus tip in the small bowel, and to the summit of colonic crypts (Moss et al., 1996, Strater et al., 1995). Few apoptotic bodies are seen in the proliferating crypt compartment (Moss et al., 1996). In contrast, in immune-mediated disorders such as celiac or inflammatory bowel disease (IBD), an increased number of enterocytes undergo premature apoptosis all along the crypt-villus axis (Moss et al., 1996, McCullen MA et al., 1997). The combination of markedly increased apoptotic and turnover rates result in architectural changes in the mucosa, characterized morphologically by villus atrophy and crypt hyperplasia. (Ruemmele and Seidman, 1998).
The mechanisms normally controlling the balance between proliferation, maturation and apoptosis in the intestine are only partially understood. There is increasing evidence that cytokines and growth factors are potent regulators of crypt cell proliferation (Podolsky, 1993, Dionne et al., 1998, Ruemmele et al., 1998). However, scant information exists concerning potential factors modulating enterocyte apoptosis under normal and pathological conditions. A major pathway resulting in apoptotic cell death is via FAS - FAS ligand interaction (Itoh et al., 1991). FAS (CD95, or APO-1) is a 48 kilodalton type I membrane protein that belongs to the tumor necrosis factor (TNF) receptor superfamily, along with the Nerve Growth Factor- and TNF-receptor, CD 27, CD 30 and CD40 (Itoh et al., 1991, Nagata and Golstein, 1995). This family is characterized by 2-6 repeats of a cysteine-rich extracellular subdomain (Nagata, 1997). The p55 TNF and FAS receptors are known to have a cytoplasmic "death domain", which induces the apoptotic response upon stimulation (Itoh et al., 1993, Tartaglia et al., 1993). The physiological ligand of FAS is a 40 kilodalton type II membrane protein (Suda et al., 1993). This FAS ligand is primarily expressed by activated, cytotoxic T-lymphocytes (Ju et al., 1994). Binding of the FAS ligand to its receptor results in apoptotic cell death upon activation of an intracellular signaling cascade, including several caspases which act on substrates such as poly-ADP-ribose-polymerase (PARP) and lamins (Tewari et al., 1995).
Normally, enterocytes are FAS positive and FAS ligand negative (Moeller et al. 1994, Strater et al., 1997). In the normal gut epithelium, FAS ligand expression has only been observed in Paneth cells (Moeller et al., 1996). The aim of the present study was to determine FAS/FAS ligand expression and its functional role in epithelial cell apoptosis and proliferation in the normal intestine. The models employed included a non-transformed

human enterocyte cell (HIEC) line (Perrault and Beaulieu, 1996) and normal colonic explant cultures.

Materials and Methods

Cell culture experiments: HIEC, human small intestinal crypt cells of fetal origin (between passages 14-20) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco), 1% glutamine and glutamax (Gibco), and 1% penicillin/streptomycin (Gibco), as previously described (Perrault and Beaulieu, 1996). Experiments on HIEC were performed in 25 cm^2 flasks (Falcon Plastics, Oxnard, CA) at a density of $3x10^5$ cells/ml in the presence or absence of recombinant human cytokines, including IFNγ (1-1000 IU/ml, Genzyme, Cambridge, MA), TNFα (1-100 ng/ml, Genzyme), or IL-1β (0.1-100 ng/ml, R+D Systems, Minneapolis, MN). Anti- IFNγ (neutralizing activity 1 ng for 1 IU IFNγ, Genzyme) or anti-TNFα antibodies (neutralizing activity 5 µg for 1 ng TNFα, Peprotech, Rocky Hill, NJ) were employed to confirm the specificity of each cytokine effect. Furthermore, agonistic anti-p55-TNF receptor antibody (R+D, 1-20 µg/ml) and antagonistic anti-p55 or anti-p75 TNF-receptor antibodies (10-50 µg/ml, R+D) were employed.

Stimulation of FAS in HIEC: To induce apoptosis, agonistic anti-FAS IgM antibody (clone CH-11, Kamiya Biomedical Co, Thousand Oaks, Ca) was used at a range of 1-500 ng/ml, alone or in combination with IFNγ (10-1000 U/ml) or TNFα (0.01-10 ng/ml) for 24-48h. In separate experiments, HIEC were incubated with IFNγ and/or TNFα. Apoptotic cell death was quantified by flow-cytometry (FACScan, Becton Dickinson, San Diego, CA) using a commercially available annexin-V-FITC kit (Apo-Alert Annxin-V kit, Clontech).

Flow cytometric analysis of FAS and FAS ligand expression on HIEC: To analyze FAS and FAS ligand expression, cytokine-stimulated or unstimulated (control) HIEC were incubated for 30 minutes at 4°C with mouse IgG anti-human FAS antibody (1 µg/ml, Pharmingen, Mississauga, ON) or mouse-IgG anti-human FAS ligand antibody (1-4 µg/ml, Pharmingen) in phosphate buffer solution (PBS) containing 2% FCS. After washing, a second fluorescein-isothiocyanate labeled anti-mouse antibody (1:100, Cappel, Aurora, OH) was added to the cells for a further 30 minutes, at 4° C. The cells were subsequently analyzed by immunofluorescence microscopy (Zeiss) and flow cytometry (B+D). At least $1x10^4$ cells were assessed for FAS and FAS ligand expression using CellQuest software (Becton Dickinson). Specificity of the staining was tested using a non-relevant mouse-IgG antibody as an isotype negative control.

Analysis of HIEC proliferation: Proliferation was measured using the ^{3}H-thymidine incorporation method, as previously described (Ruemmele et al., 1998). Briefly, subconfluent HIEC were stimulated with EGF (50 ng/ml) and/or anti-FAS IgM antibody (1-500 ng/ml) for 20h in the presence of 1% FCS. During the final 4h, ^{3}H-labeled thymidine (1µCi/ml) was added. Thereafter the cells were washed, harvested, and radioactivity was measured (counts per minute). Results were expressed as percentage of ^{3}H-thymidine incorporation relative to control wells without stimulation.

Organ culture experiments: Normal colonic biopsies (n=5) from 12 consenting patients (mean age: 12.3, range: 6-15 years) were obtained during their diagnostic work-up for recurrent abdominal pain. The tissue was cultured at 37°C in a humidified atmosphere containing 5% CO_2 in CMRL-1066 medium (Sigma) supplemented with 20mM TRIS buffer, 1% penicillin/streptomycin, 0.1% gentamycin, 0.05% amphotericin, and 10% FCS. The explants were incubated in the presence or absence (control cultures) of IFNγ (10-100 U/ml) or TNFα (10-100 ng/ml), alone or in combination. To confirm the specificity of each cytokine's effect, neutralizing anti-IFNγ and anti-TNFα antibodies were used, as above. After 20 hours, the cultured biopsies were embedded in gelatine and snap frozen.

Immunohistochemistry for FAS and FAS ligand in colonic organ cultures: After cutting and fixation in acetone, thin sections were processed for immunohistochemical staining of FAS or FAS ligand. Endogenous peroxidase activity was blocked by adding 0.3% H_2O_2. Thereafter, the slides were incubated with either anti- FAS antibody (1:100-10.000) or anti-FAS ligand antibody (1:10-1:1000) in PBS, followed by a biotinylated anti-immunoglobulin antibody (Universal Secondary Antibody, Immunon, Pittsburgh, PA), and a third streptavidin-conjugated antibody with peroxidase activity (Immunon). The specific labeling was visualized using 3,3'-diamino-benzidine (DAB)-H_2O_2, which results in an intense brown color. A non-relevant mouse-IgG antibody was used as an isotype control to determine any background staining.

To quantify FAS expression on intestinal epithelial cells, a serial dilution technique was employed, as described by Pearse (1980). The specific antibody was serially diluted (1:100-1:10,000) until the characteristic staining almost disappeared under control conditions. FAS expression was then compared at the highest dilution (1:10,000) between stimulated and unstimulated tissues by two investigators, blinded to the experimental conditions. A score was assigned from 0 to 3, where 0= absent staining, 1= focal staining with less than 30% of epithelial cells positive, 2= 30-95% positive cells, and 3= all

epithelial cells positive.

Stimulation of FAS in colonic organ cultures: To induce colonocyte apoptosis, the agonistic anti-FAS-CH11 antibody was used at a concentration of 50-500 ng/ml alone, or in combination with IFNγ (10-100 U/ml) for 8-16h. To localize which intestinal mucosal cells underwent FAS-induced apoptosis, *in situ* TUNEL staining was performed using a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, freshly frozen explant cultures were treated with proteinase K (20 μg/ml in 10 mM TRIS-Cl, pH 7.6) for 30 min., washed with PBS, and endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. Thereafter, the sections were permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate. To label specific DNA-strand breaks, the sections were incubated with the label mixture containing fluorescein-dUTP and the enzyme terminal deoxynucleotidyl transferase (TdT) in a humidified chamber for 60 min at 37°C. Specific labeling was visualized by a sheep anti-fluorescein antibody conjugated with horse-radish peroxidase at 37°C in a humidified chamber for 30 min. Following washing, color development was performed by incubating sections with a DAB-H_2O_2-solution for 15 min. Since the TUNEL technique may falsely label DNA strand breaks in proliferating or necrotic cells, only TUNEL-positive cells with morphological characteristics of apoptosis were considered to be positive.

Statistical analysis and ethical considerations: All experiments were carried out in duplicate. The values expressed are the mean ± SD of 5 sets of experiments. Non-parametric results were compared by the two-tailed Mann-Whitney U-test. Differences with $p < 0.05$ were regarded as significant. The study protocol and consent forms for additional rectal biopsies were approved by the Ethics Committee of Ste-Justine Hospital.

Results

The role of FAS in HIEC apoptosis

HIEC constitutively expressed FAS, as analyzed by immunofluorescence microscopy and flow cytometry. The functional role of FAS was studied using agonistic IgM anti-FAS (1-500 ng/ml), an antibody which induces apoptosis analogous to the FAS ligand route. Stimulation of FAS did not induce HIEC apoptosis, under all experimental conditions tested (Fig.1a). However, coincubation with IFNγ and/or TNFα and IgM anti-FAS antibody markedly increased the susceptibility of HIEC to FAS-induced apoptosis. IFNγ, alone without apoptotic effect, increased FAS-induced HIEC apoptosis in a dose-dependent manner reaching a maximum of 31±3.3% after a 24h incubation period (Fig.1a). Similarly, costimulation with low concentrations (0.01-10 ng/ml) of TNFα, which alone only slightly induced HIEC apoptosis, increased the apoptosis rate in response to anti-FAS (Fig.1b). A particularly potent sensitizing effect of HIEC to FAS-stimulation was observed with the combination of both cytokines, as demonstrated in Fig1b. Furthermore, IFNγ also rendered HIEC highly susceptible to TNFα-induced apoptosis. Therefore, the high rate of apoptosis (≥80% after 24h) observed with IFNγ (100 U/ml) and TNFα (10 ng/ml) in the presence of anti-FAS IgM (100 ng/ml) was likely an additive effect of TNFα- and anti-FAS antibody induced apoptosis. Morphological analysis by immunofluorescence revealed the typical signs of apoptosis, including nuclear condensation and fragmentation, in parallel with cell shrinkage and the formation of apoptotic bodies (not shown). Addition of the corresponding neutralizing anti-cytokine antibody to the combination of IFNγ or TNFα and anti-FAS antibody reduced the percentage of apoptotic cells to that observed under control conditions.

Cytokine regulation of FAS-expression on HIEC

In order to uncover the mechanisms underlying cytokine-induced sensitization of HIEC to FAS-mediated apoptosis, their modulation of FAS expression was analyzed by flow cytometry. Stimulation with IFNγ markedly upregulated FAS expression (Fig.2a), at concentrations as low as 10 U/ml (159±19% expression compared to unstimulated conditions). A plateau effect was observed at 100 U/ml IFNγ, with maximal FAS stimulation of 190±13% ($p<0.005$). Similarly, TNFα significantly upregulated FAS expression at concentrations as low as 1 ng/ml (Fig.2a), with maximal effect at 10 ng/ml (174±12% of control). FAS expression was maximally stimulated by either cytokine alone after 24h.

The combination of IFNγ and TNFα resulted in synergistic enhancement of FAS

expression (Fig.2b). Doses as low as 1 U/ml IFNγ combined with 1 ng/ml TNFα markedly increased FAS surface expression on HIEC. A maximal effect (348%) was achieved after 24h stimulation with 100 U/ml IFNγ and 10 ng/ml TNFα ($p<0.005$ vs. unstimulated control cells). IL-1β failed to up- or down- regulate FAS expression on HIEC, at all doses tested. Furthermore, the combination of IL-1β and IFNγ did not alter the stimulatory effect of IFNγ on FAS expression. The specificity of each cytokine's effect was confirmed using neutralizing anti-cytokine antibodies, which abolished the FAS-stimulatory effect of IFNγ and TNFα.

TNFα upregulates HIEC FAS expression via the p55-TNF receptor 1

Incubation with the agonistic antibody to the TNF-R1 (5-20μg/ml) increased HIEC FAS expression (143±20% versus control) similar to the effect of TNFα (168%). Furthermore, the combination of IFNγ (100 U/ml) and the anti-p55 TNF-R1 agonistic antibody (10μg/ml) resulted in a marked increase in FAS expression (273±28%) compared to unstimulated control cells (Fig.2c) ($p<0.005$). This effect was only slightly lower than that observed with the combination of IFNγ and TNFα. Preincubation of the cells with an antagonistic anti-p55 TNF-R1 antibody abolished the effect of TNFα (103%± 17, $p<0.01$). On the other hand, the antagonistic anti-p75-TNF-R2 antibody did not alter TNFα induced FAS upregulation.

HIEC do not express FAS ligand

Assessment of FAS ligand status by flow cytometry revealed that HIEC do not express this protein under basal conditions, nor was it inducible by any of the cytokines tested, alone or in combination. Under similar experimental conditions, positive FAS ligand expression was observed in PMA-stimulated lymphocytes from a healthy donor.

The role of FAS in HIEC turnover

To study the potential involvement of FAS in enterocyte proliferation and turnover, HIEC were stimulated for 20h with the agonistic IgM anti-FAS antibody (1-500 ng/ml). DNA synthesis was subsequently measured using the ^{3}H-thymidine incorporation method. No change in HIEC proliferation was observed after FAS-stimulation (Fig.3). At maximal doses, a weak growth inhibitory effect was noted (91±5% of control proliferation, p=ns). Furthermore, the growth-promoting effect of EGF (50 ng/ml, 254±22% versus control) was not altered by simultaneous stimulation of FAS (1-500 ng/ml).

Regulation of FAS expression in colonic organ cultures

Intestinal epithelial cells in normal colonic biopsies were also found to express FAS. The signal was intense in the cytoplasm, particularly on the basolateral surface. At the apical side, only weak FAS staining was observed in cells near the luminal surface. Intensity of FAS expression was region dependent, with the lowest expression on immature crypt cells, gradually increasing towards the crypt summit. The expression of this receptor was unaltered after *ex vivo* organ culture of the biopsies for periods ranging from 6 to 20h. FAS expression of colonocytes in organ cultures was slightly increased by IFNγ (10-100 U/ml) or TNFα (10-100 ng/ml). The combination of IFNγ (100 U/ml) and TNFα (10-100 ng/ml) was particularly potent in upregulating FAS expression on mature, as well as immature colonocytes (Fig.4). Preincubation of explants with either cytokine and the corresponding neutralizing antibody abolished this effect. To quantify increased FAS expression, the specific anti-FAS antibody was serially diluted. At a concentration of 1:10,000, the specific staining disappeared almost completely in unstimulated biopsies (mean score 0.2). However, in IFNγ and TNFα treated biopsies, intestinal epithelial cells remained FAS positive (mean score: 1.4 [for IFNγ (100 U/ml)+TNFα (100 ng/ml)], mean score: 0.8 [IFNγ (100 U/ml)+TNFα (10 ng/ml)]) at the same antibody-dilution (1:10,000).

FAS-induced apoptosis in colonic organ cultures

To study the functional role of FAS, cultures were stimulated with the agonistic IgM anti-FAS antibody (50-500 ng/ml) for up to 16h in the presence or absence (control) of pro-inflammatory cytokines and analyzed by *in situ* TUNEL-staining. A high degree of intestinal epithelial cell apoptosis was observed after stimulation of FAS with anti-FAS CH11 at 500 ng/ml. Lower concentrations of this FAS-agonist (50-100 ng/ml) did only slightly incude apoptotic cell death of colonocytes at the crypt summit (Fig.5). However, costimulation with the pro-inflammatory cytokine IFNγ 100 U/ml and IgM anti-FAS antibody markedly increased the susceptibility of colonocytes to FAS-induced apoptosis. In the presence of these cytokines, low FAS-agonist doses (50-100 ng/ml), alone with minimal effect, induced a high degree of intestinal epithelial cell apoptosis. Although other mucosal cells in the lamina propria were also positive by TUNEL staining, the epithelial cells were severely affected, leading to marked epithelial destruction (Fig.5). IFNγ alone failed to induce intestinal epithelial cell apoptosis, as shown by TUNEL-assay (Fig.5).

Colonocytes do not express FAS ligand in organ cultures

Immunohistochemical analysis of FAS ligand expression confirmed that normal colonic epithelial cells were negative for this protein. Furthermore, FAS ligand was not inducible

by IFNγ or TNFα, alone or in combination, in all experimental conditions tested. In the lamina propria, few FAS ligand positive lymphocytes were noted.

Discussion

Intestinal morphology and function are considerably altered in immune-mediated bowel diseases. This results in part from the markedly increased rates of epithelial cell loss via apoptosis and hastened cell turnover, accompanied by a preponderance of incompletely differentiated enterocytes (MacDonald and Spencer, 1988, Ruemmele et al., 1998). Recent studies suggest that the augmented apoptosis is mediated via FAS-FAS ligand interaction. A marked increase in FAS ligand expressing activated lamina propria lymphocytes was observed in actively inflamed tissue from ulcerative colitis. These activated T-cells, in close proximity to the epithelial basal membrane, may induce apoptosis in enterocytes via FAS-FAS ligand interaction, leading to the observed epithelial damage (Strater et al., 1997). Recently, Sakai et al. (1997) demonstrated that FAS-mediated cytotoxicity is also involved in the pathogenesis of the enteropathy in a murine model of graft-versus-host disease.
This is the first report showing that HIEC, a cell model of non-transformed human enterocytes, are resistant to FAS-induced apoptosis, despite being FAS positive. In colonic explant cultures however, FAS-engagement clearly induced intestinal epithelial cell apoptosis, in a dose-dependent manner. These results are in keeping with those obtained in isolated human colonic crypts, recently reported by Strater et al. (1997). However, in our model, higher anti-FAS doses were required to induce colonocyte apoptosis. This might be due to the relatively normally preserved tissue architecture of explant cultures, compared to the isolated crypts used by Strater et al (1997). It was recently shown that enterocytes rapidly undergo apoptosis after isolation, due to loss of contact with the basal membrane (Grossmann et al., 1998). Alternatively, the resistance of HIEC to FAS-induced apoptosis may be more a characteristic of enterocytes from the small bowel, since they are of ileal origin (Perrault and Beaulieu, 1996). Moreover, Merrit et al. (1995) recently showed that there is a marked difference in the distribution of pro- and anti-apoptotic proteins of the bcl-family between the small and large bowel. This might partially explain the different susceptibility of enterocytes and colonocytes to undergo apoptosis upon FAS-activation. The observation that in the absence of inflammation, FAS-induced epithelial cell apoptosis was moderate in colonic organ cultures supports the hypothesis that costimulatory factors, or high doses of anti-FAS antibodies, are needed to activate the intracellular signaling cascade that results in apoptosis. We found that, upon stimulation with the pro-inflammatory cytokines IFNγ and TNFα, both HIEC and colonocytes in explants became highly susceptible to FAS-induced apoptosis *in vitro*. In both models, one of the key molecular mechanisms underlying this sensitizing effect of cytokines to FAS-mediated apoptosis was via up-regulation of this death receptor. Our results furthermore indicate that

similar mechanisms of FAS regulation seen at the single cell level may be relevant in the complex network of intercellular interactions, present in the inflamed intestinal mucosa.
FAS upregulation in enterocytes was not a general feature of proinflammatory cytokines, as IL-1β failed to modulate FAS expression at all experimental conditions tested. IL-1β has been reported to induce FAS down-regulation in synovial cells (Tsuboi et al., 1996).
The data presented in this study also revealed that the TNFα effect was mediated via the p55 TNF-receptor. Stimulation of this receptor with an agonistic antibody resulted in upregulation of FAS to an extent similar to that observed with TNFα. The dominant role of the p55 TNF-R1 was confirmed using a selective antagonistic antibody to the p55 TNF-receptor, which abolished the TNFα induced FAS upregulatory effect. Selective blockade of the p75 TNF-receptor had no effect.
Cytokine induced FAS upregulation on normal enterocytes is of particular interest to the pathogenesis of IBD, where increased gene expression and production of pro-inflammatory cytokines, such as IL-1β, IFNγ and TNFα have been observed (Fais et al., 1991. Dionne et al., 1997). We observed a markedly increased apoptosis rate after co-stimulation with IFNγ and/or TNFα in the presence of an anti-FAS agonist, compared to anti-FAS antibody alone. This suggests that *in vivo*, the susceptibility of enterocytes to FAS-induced apoptosis can be markedly enhanced by locally secreted cytokines in zones of active inflammation. Itoh et al. (1993) were the first to demonstrate that IFNγ treatment of the HT29 colon tumor cell line enhanced FAS gene expression, resulting in increased FAS expression. Furthermore, apoptosis via FAS-FAS ligand interaction was only observed after IFNγ pretreatment of HT29 cells, in concert with our results (Abreu-Martin et al., 1995, Ossina et al. 1997). Koshiji et al. (1998) confirmed the importance of costimulatory factors to induce apoptosis in the COLO 201 intestinal cancer cell line. TNFα as well as IFNγ were potent upregulators of FAS-expression. Simultaneously, these cytokines down-modulated bcl-2, an anti-apoptotic protein, and upregulated the pro-apoptotic protein bax in this transformed cell line. These findings indicate that additional mechanisms may be involved in cytokine-regulation of sensitization to FAS. Keane et al. (1996) showed that distinct intracellular signaling events, rather than the upregulation of FAS, account for IFNγ-sensitizing to FAS-induced apoptosis in breast epithelial cancer cells. In that study, IFNγ-induced upregulation of caspase-1 was critical in triggering apoptosis upon FAS-engagement. More recently, Ossina et al. (1997) markedly extended our understanding of this important cytokine effect. IFNγ induced HT-29 cell expression of various pro-apoptotic genes, especially caspases 1,3,4,7,8,10 and the pro-apoptotic protein bak. We therefore speculate that, in addition to FAS-receptor upregulation, similar molecular events contributed to the observed cytokine-sensitization to FAS-induced apoptosis in the normal

intestinal epithelial cells employed in our study.
The physiological and functional role of FAS on immature and mature enterocytes is not yet fully understood. A regulatory role in intestinal epithelial cell turnover, perhaps via auto-or paracrine FAS-FAS ligand interaction, was recently proposed (Averil, 1997). Under basal conditions, enterocytes are FAS ligand negative. To determine whether cytokines can induce the expression of this protein on enterocytes, we stimulated normal human intestinal epithelial cells with up to supraphysiological doses of IFNγ and TNFα. In contrast to transformed malignant colonocytes which can be FAS ligand positive (O'Connell J et al., 1996), no FAS ligand expression was detected, nor was it inducible in normal epithelial cells, as shown herein with HIEC and intestinal epithelial cells in colonic organ cultures. However, using normal colonic tissue, Iwamoto et al. (1996) recently observed weak FAS ligand expression at the basolateral surface of mature intestinal epithelial cells. They were simultaneously FAS positive. In addition, most epithelial cells were FAS ligand positive in actively inflamed colonic tissue obtained from UC patients, (Iwamoto et al. 1996). Furthermore, some of these cells were found to undergo apoptosis, as shown by TUNEL-staining. In contrast, other studies with human colonic and mouse small bowel tissue did not confirm that non-transformed intestinal epithelial cells express FAS ligand (Ueyama et al. 1998, Inagaki-Ohara et al., 1997), even in the presence of inflammation. These reports are in keeping with our findings. Although our data do not support the hypothesis of an auto- or paracrine mechanism controlling enterocyte apoptosis, other factors, yet to be described, may be involved.
FAS may play an important role in the turnover of tissues with a high proliferation rate. In some leukemic cell lines for example, stimulation of FAS results in an increased mitogenic activity (Mapara et al., 1993). However, no pro- or anti-mitogenic effect was seen in HIEC upon FAS-stimulation. Our data suggest that this receptor is not likely to be implicated in the physiological regulation of normal intestinal epithelial cell proliferation.
In summary, our data show that certain pro-inflammatory cytokines, potentially released locally from activated immune cells, upregulate basal FAS expression on normal human intestinal epithelial cells, thereby rendering these cells susceptible to FAS-induced apoptosis. These results are in concert with reports describing increased FAS expression on enterocytes in the actively inflamed mucosa in CD or UC (Iwamoto et al., 1996, Reich et al., 1996). Specifically, IFNγ and TNFα are cytokines capable of upregulating the expression of this receptor on non-transformed intestinal epithelial cells. Our data establish that costimulatory factors released locally, such as cytokines, can markedly increase the susceptibility of enterocytes to FAS-FAS ligand induced apoptosis. The present study thus reveals a novel aspect among the increasing number of important interactions between

immune cells and enterocytes that are mediated via cytokines (Ruemmele and Seidman, 1998).

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Figure legends

Fig. 1: Effect of the proinflammatory cytokines IFNγ and/or TNFα on FAS-induced apoptosis in HIEC. a) Isolated stimulation of FAS with the agonistic anti-FAS CH11 antibody (1-500 ng/ml) failed to induce HIEC apoptosis, determined by Annexin-V assay, as described in Materials and Methods. However, IFNγ (1-500 U/ml, x24h), alone without effect, as well as TNFα (0.01-10 ng/ml, x24h) significantly enhanced the susceptibility of HIEC to undergo apoptosis upon stimulation of FAS (100 ng/ml). b) A particularly potent sensitizing effect to undergo apoptosis in response to FAS engagement was observed with the combination of IFNγ (100 U/ml) and TNFα (0.1-10 ng/ml), which alone induced a relatively high degree of apoptosis.

Fig. 2: Cytokine regulation of FAS expression on HIEC, analyzed by flow cytometry as described in Materials and Methods. a) Treatment with IFNγ (black bars) or TNFα (grey bars) markedly increased FAS expression, whereas IL-1β had no effect (white bars). b) The combination of IFNγ and TNFα synergistically increased FAS expression, in a dose-dependent manner. c) Receptor studies revealed that the effect of TNFα on FAS was mediated via the p55 TNF receptor. Selectively blocking the p55 TNF receptor reversed TNFα induced FAS upregulation, whereas inhibition of the p75 TNF receptor failed to alter the TNFα effect. *p<0.01 (versus control).

Fig. 3: HIEC proliferation in response to FAS stimulation. Stimulation of FAS did not significantly alter HIEC growth, as determined by ^{3}H-thymidine incorporation (see Materials and Methods). The mitogenic effect of EGF was unaltered by the simultaneous stimulation of FAS. *p<0.005.

Fig. 4: FAS expression on epithelial cells in colonic organ cultures. Shown are representative micrographs of colonic crypt summits. At a 1:10,000 dilution, the anti-FAS antibody staining of enterocytes was almost absent in untreated colonic explants (a). At the same anti-FAS antibody concentration, IFNγ and TNFα treated biopsies were highly FAS positive (brown color). b). IFNγ (100 U/ml) and TNFα (100 ng/ml), c) IFNγ (100 U/ml) and TNFα (10 ng/ml). Original magnification x400.

Fig. 5: FAS induced apoptosis in colonic organ cultures, analyzed by *in situ* TUNEL-staining. In unstimulated control cultures, only few apoptotic cells were observed at the crypt summit (brown color, condensed/fragmented nuclei). Incubation with IgM anti-FAS

antibody (100 ng/ml, x 16h) did only slightly induce colonocyte apoptosis at the crypt summit. Co-stimulation of explant cultures with anti-FAS CH11 antibody (100 ng/ml) and IFNγ (100 U/ml), alone without effect, markedly increased colonocyte susceptibility to FAS-induced apoptosis, with occurrence of apoptotic cells all along the crypts, leading to significant epithelial destruction. Staining is also seen in the lamina propria.

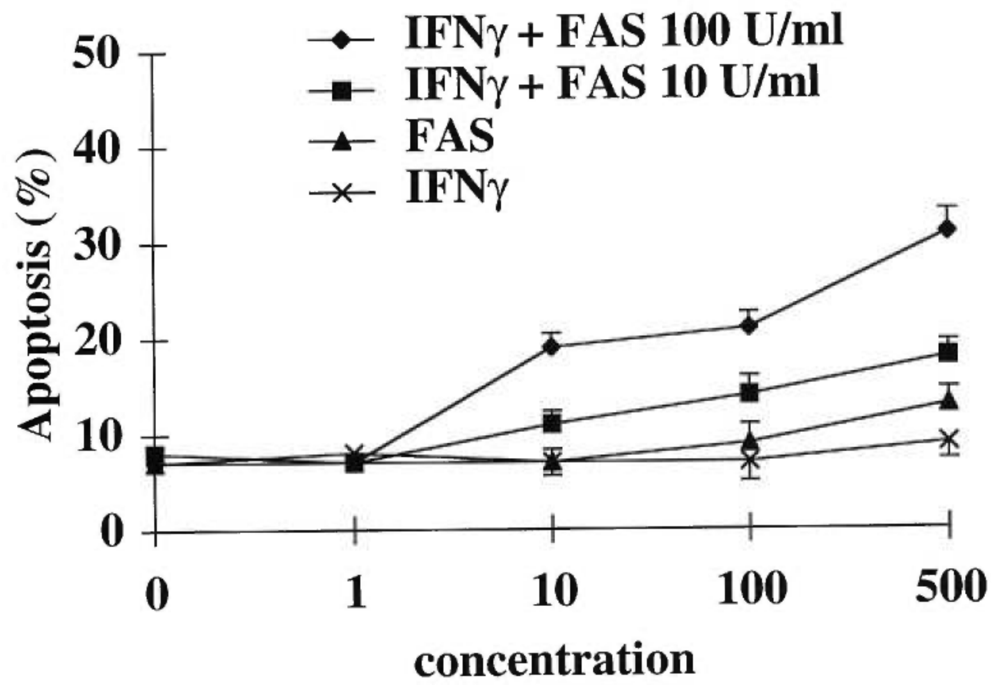


Fig.1a

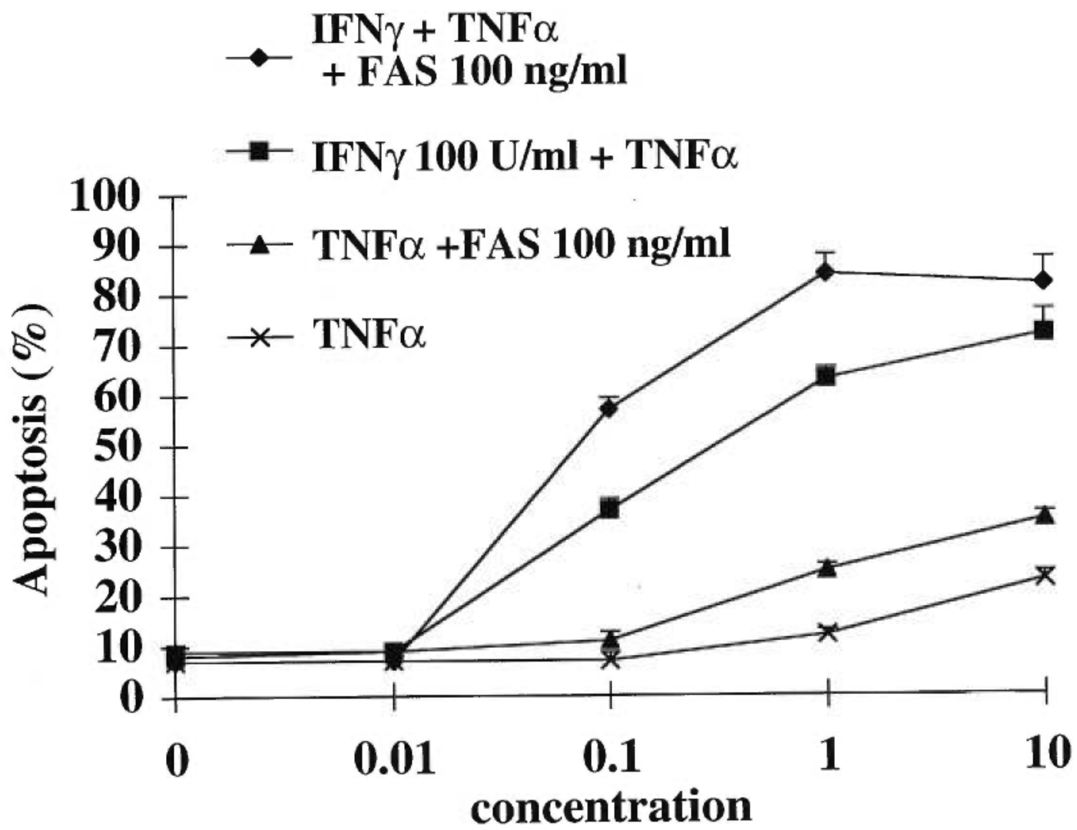
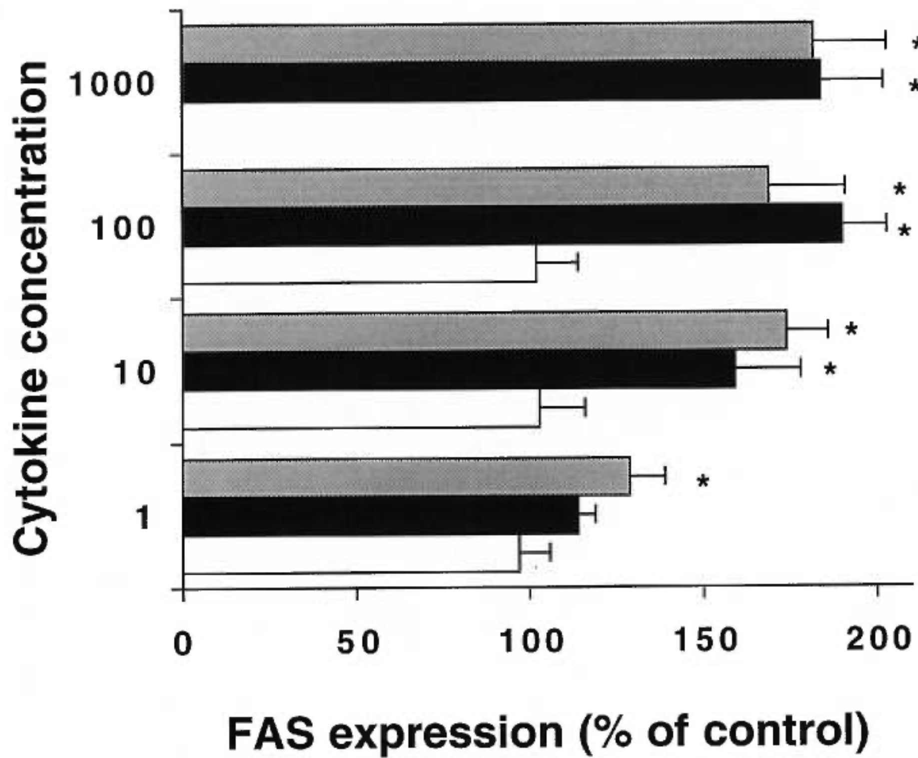


Fig.1b



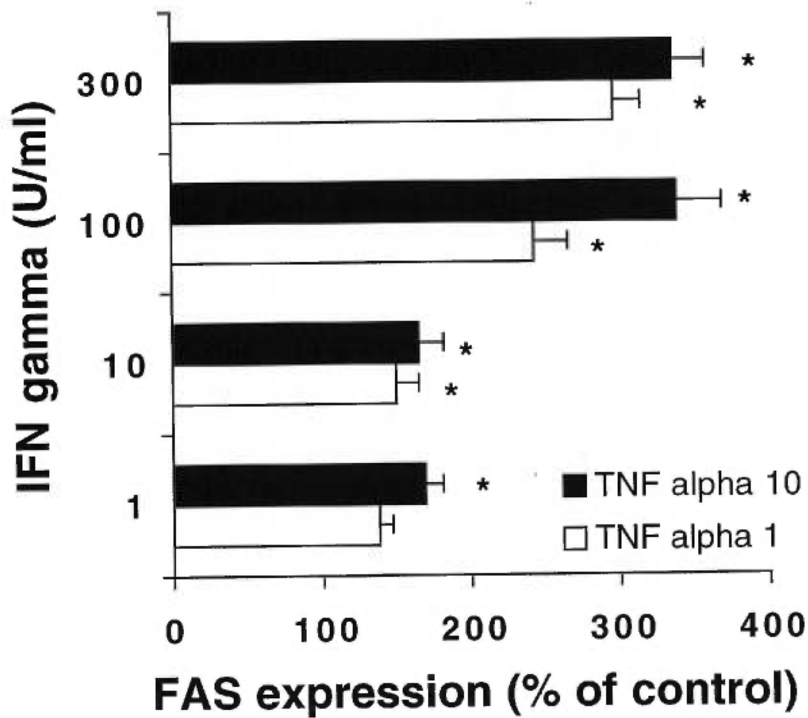


Fig.2a,b

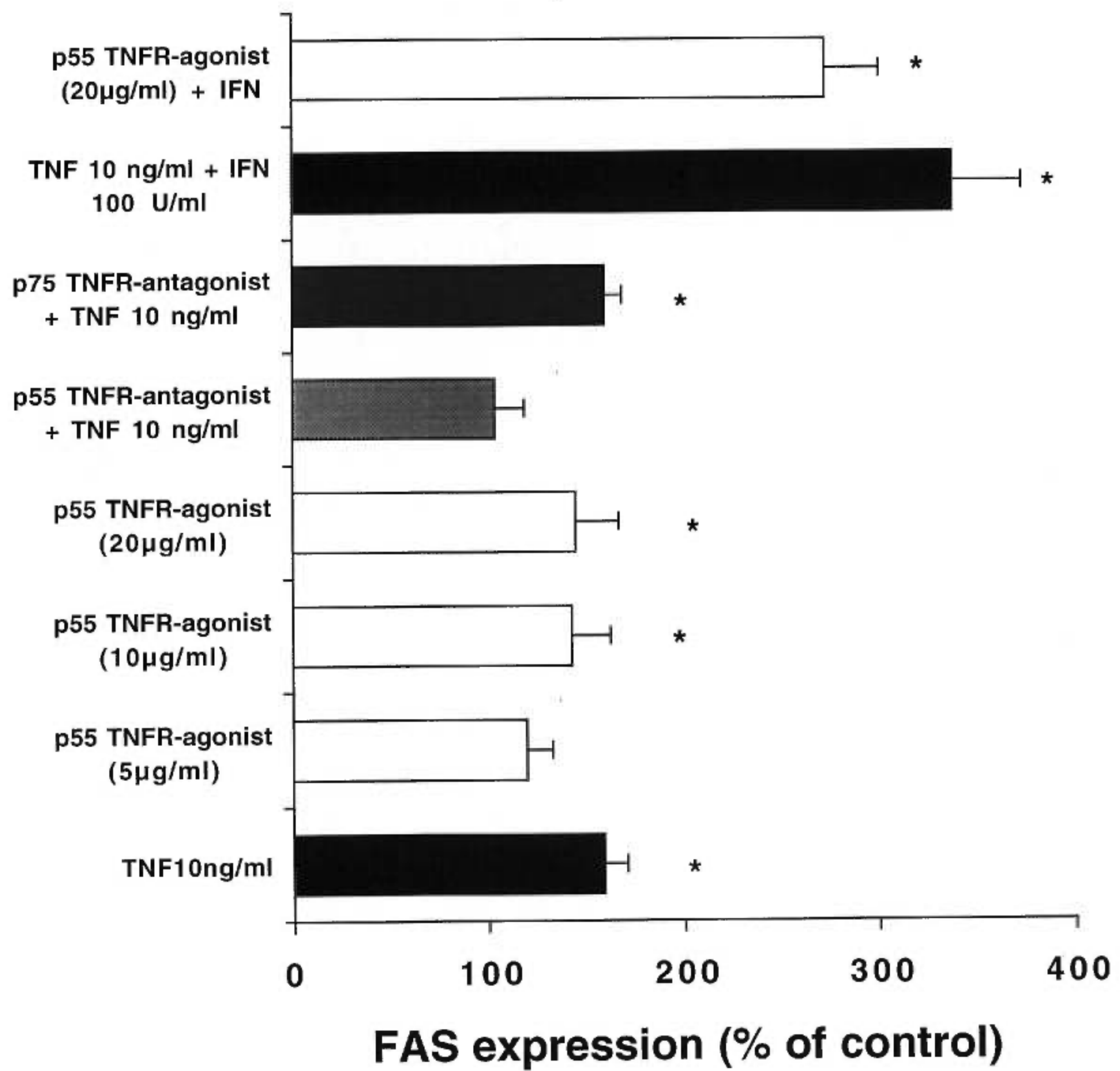


Fig.2c

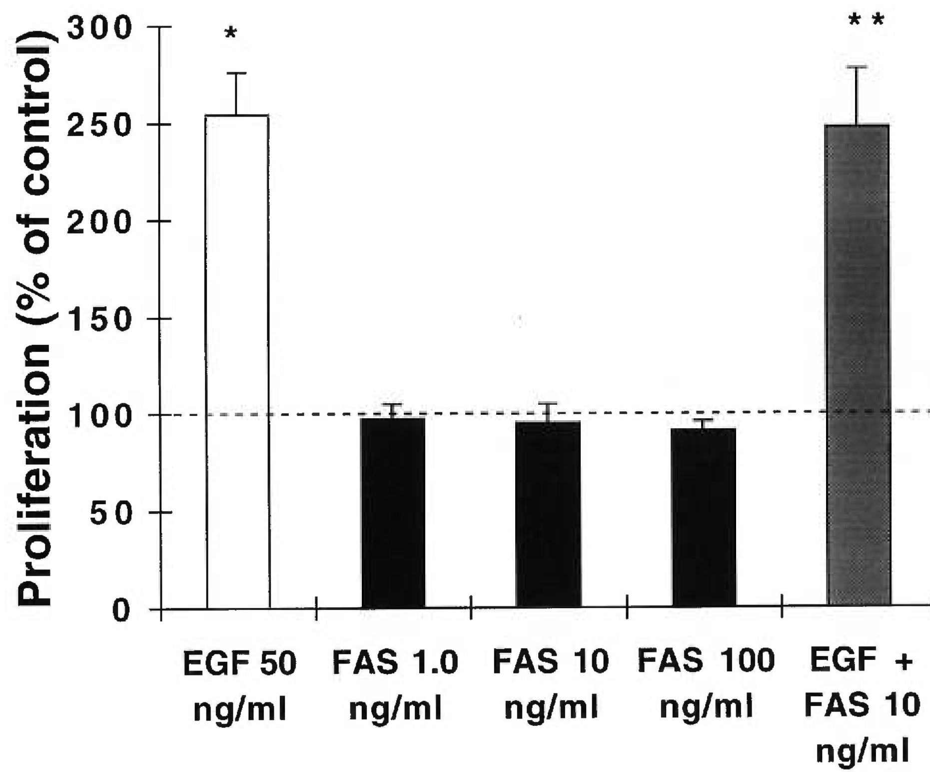


Fig.3

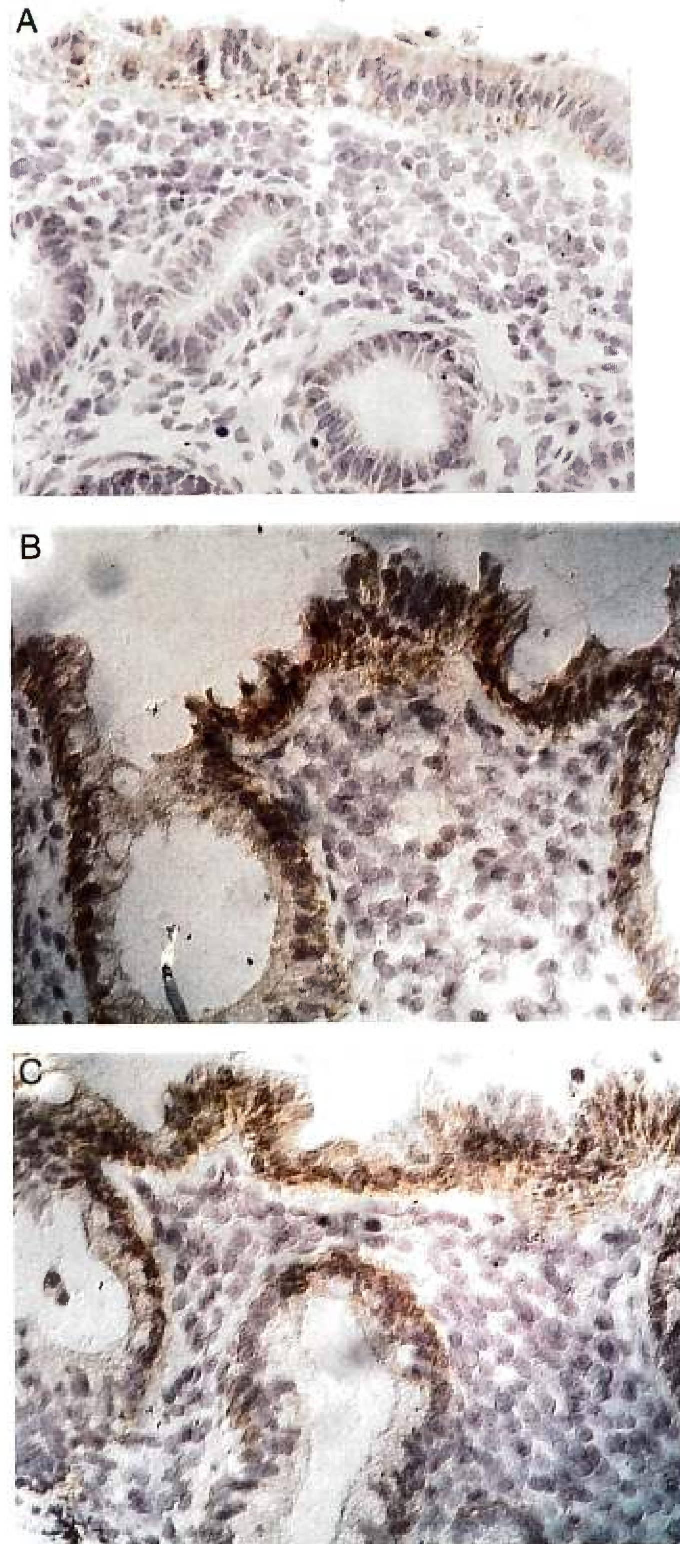


Fig.4

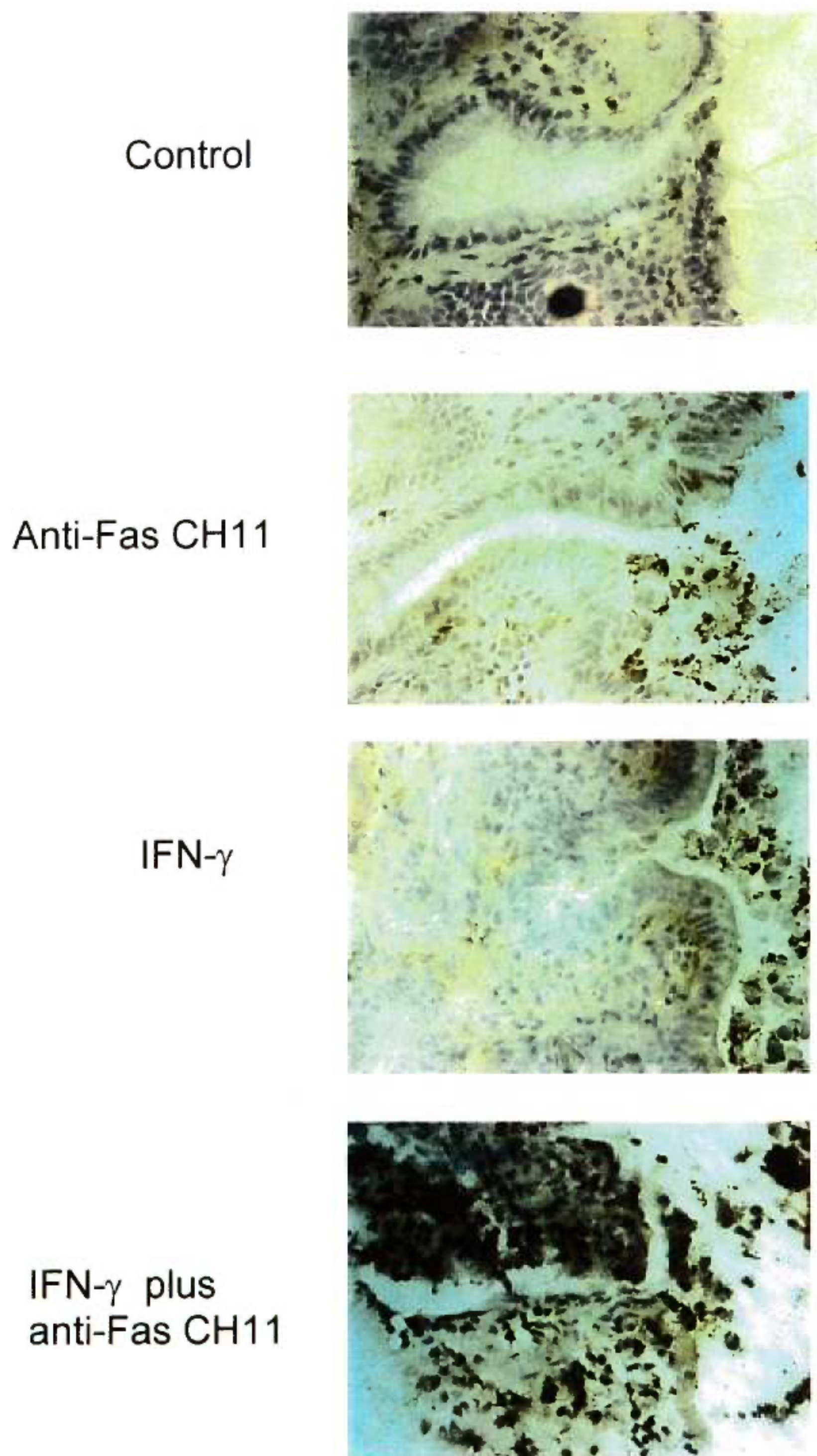


Fig.5

BUTYRATE MEDIATES CACO-2 CELL APOPTOSIS VIA UP-REGULATION OF PRO-APOPTOTIC BAK AND INDUCING CASPASE-3 MEDIATED CLEAVAGE OF POLY-(ADP-RIBOSE) POLYMERASE (PARP)

Running Title: Molecular mechanisms of butyrate induced apoptosis

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ABSTRACT

Butyrate exerts potent anti-tumor effects by inhibiting cancer cell growth and inducing apoptosis. However, the molecular mechanisms mediating these effects remain largely unknown. Using the Caco-2 cell line, a well established model of colon cancer cells, our data show that butyrate induced apoptosis (maximum 79%) is mediated via activation of the caspase-cascade. A key event was the proteolytic activation of caspase-3, triggering degradation of poly-(ADP-ribose) polymerase (PARP). Inactivation of caspase-3 with the tetrapeptide zDEVD-FMK completely inhibited the apoptotic response to butyrate. In parallel, butyrate potently up-regulated the expression of the pro-apoptotic protein bak, without changing Caco-2 cell bcl-2 expression. Butyrate-induced Caco-2 cell apoptosis was completely blocked by the addition of cycloheximide, indicating the necessity of protein synthesis. However, when this inhibitor was added at a time point where bak expression was already enhanced (12-16h after butyrate stimulation), it failed to protect Caco-2 cells against apoptosis. Taken together, these data provide evidence that the molecular events involved in butyrate induced colon cancer cell apoptosis include the caspase-cascade and the mitochondrial bcl-pathway.

Key words: colon cancer, apoptosis, Caco-2 cells, butyrate, short chain fatty acid, caspase, bcl-2, bak.

INTRODUCTION

Short chain fatty acids, particularly butyrate, are thought to play an important role in preventing the development of colon cancer (Hague et al. 1993, Giardina et al. 1998 Velazquez et al. 1996). An inverse relationship between colon tumor mass and fecal butyrate levels was observed in rats, supporting this hypothesis (McIntyre et al. 1993). Various mechanisms have been proposed to explain how this particular short chain fatty acid inhibits tumorigenesis. Butyrate has been shown to inhibit cancer cell growth, leading to differentiation (Toscani et al. 1988, Kim et al. 1980, Whithead et al. 1986). Furthermore, butyrate is a potent inducer of apoptosis in cancer cells *in vitro* (Medina et al. 1998, Calabresse et al. 1993). Recently, Dolara et al. (1998) provided evidence that oral administration of butyrate in form of an enteric-resistant slow-release pellet significantly increased colonocyte apoptosis rate in a rat model of colon cancer, indicating a potential anti-cancer effect *in vivo.*

Over the past few years, major advances have been made in our understanding of the molecular mechanisms that trigger apoptosis. The regulation of apoptotic cell death is often altered in transformed cells. The most common mutations in colon cancers cells are not surprisingly found among genes implicated in the regulation of apoptosis, including the p53 gene (el-Mahdani et al. 1997).An important pathway leading to apoptotic cell death is via the activation of the intracellular cascade of cysteine proteases, now referred to as caspases (Alnemri et al. 1996). The recruitment of cytoplasmic death domains to activated membrane receptors, such as the p55 TNF-receptor or FAS, results in the activation of these caspases, which are synthesized as zymogens. This cascade is thought to represent a major regulatory step in the apoptotic pathway. One key endpoint in this cascade is activation of caspase-3, which cleaves several substrates such as the DNA-repair enzyme poly (ADP-ribose) polymerase (PARP) or DNA-fragmentation factor (DFF 45), leading to the typical 180 bp-DNA strand breaks observed in the course of apoptosis. Recently Medina et al. (1997) put forward evidence that butyrate induced apoptosis in cancer cells is triggered via activation of the caspase cascade.

In this study, the mechanisms of butyrate-induced apoptosis were studied using the Caco-2 colon cancer cell line. Butyrate induced apoptosis required the activation of caspase-3 and was completely abolished by specific caspase-3 inhibition. Rapid cleavage of PARP by caspase-3 was observed in apoptotic Caco-2 cells in response to butyrate. Inhibition of protein synthesis by cycloheximide blocked the apoptotic effect of butyrate, indicating the necessity of protein-neosynthesis to execute apoptosis. Cycloheximide also inhibited the activation of caspase-3. Furthermore, butyrate induced upregulation of the pro-apoptotic protein bak in apoptotic Caco-2 cells was also suppressed by cycloheximide.

RESULTS

Effect of butyrate on Caco-2 cell proliferation

Butyrate inhibited Caco-2 cell proliferation in a dose-dependent manner, as shown in Fig.1. This effect was observed at concentrations as low as 0.1 mM (89±3% versus control, p<0.01). Maximal inhibition by butyrate was noted at a dose of 10 mM (62±8% versus control, p<0.01).

Effect of butyrate on Caco-2 cell apoptosis

Under control conditions, near-confluent Caco-2 cell monolayers showed a spontaneous apoptosis rate of 9±3% after 24 h, which gradually increased to 18±6% at 72 h. Incubation with butyrate strongly induced Caco-2 cell apoptosis in a time- and dose-dependent manner, with an initial lag-phase of 14-16 h. A maximal apoptotic response of 79±12% was observed with 100 mM butyrate after 24 h incubation (Fig.2a). Almost the same effect (76±8%) was seen with 10-fold lower butyrate-concentrations when the incubation period was prolonged to 48 h.

Butyrate-treated cells showed the typical morphological characteristics of apoptosis when analyzed by immunofluorescence. After staining with the DNA-dye HOECHST 33342, nuclear condensation and fragmentation were observed, as were the formation of apoptotic bodies (Fig.2b). Incubation of butyrate-treated Caco-2 cells with FITC-labeled annexin V revealed the presence of phosphatidyl serine on the external leaf of the plasma membrane (Fig.2c), an event characteristic of early apoptosis.

Role of protein-synthesis in butyrate induced apoptosis

Butyrate induced Caco-2 cell apoptosis was observed to be dependent on protein synthesis. Simultaneous incubation of Caco-2 cells with butyrate and the protein synthesis inhibitor cycloheximide (100 µg/ml) markedly suppressed the pro-apoptotic effect of the short chain fatty acid (Fig.3a). To further investigate the role of protein neosynthesis on butyrate induced Caco-2 cell apoptosis, cycloheximide was added at various time points after stimulation with butyrate. As shown in Fig.3b, butyrate-induced apoptosis was completely blocked if cycloheximide was added within 8h of butyrate stimulation. Thereafter, the protective effect of cycloheximide was progressively less, with no significant protective effect if added 14h or later after butyrate-stimulation.

Activation of caspase-3 and subsequent degradation of PARP during butyrate-induced Caco-2 cell apoptosis

Western blot analysis of butyrate treated Caco-2 cells revealed cleavage of the 32 kDa pro-enzyme caspase-3 to its active 17 kDa form (Fig.4a). Butyrate (5-100 mM) strongly activated this caspase, whereas lower doses failed to do so, consistent with the observation

of a lack of an apoptotic response at lower butyrate concentrations. In the presence of cycloheximide, no activation of caspase-3 was observed at all butyrate-doses tested (Fig.4b), indicating that protein synthesis was required upstream to activate the caspase-cascade. In a second step, we assessed the degradation of the DNA-repair enzyme PARP, the known substrate of caspase-3. As shown in Fig.4c, minimal PARP-degradation was observed in unstimulated Caco-2 cells, indicating slight spontaneous apoptotic activity. Incubation with increasing doses of butyrate resulted in marked degradation of PARP, seen as a typical 89 kDa band, which was almost complete at butyrate-concentrations of 100 μM.

Inhibition of butyrate-induced Caco-2 cell apoptosis by zDEVD-FMK

The role of PARP degradation by caspase-3 in butyrate-induced Caco-2 cell apoptosis was further examined using the caspase-3 inhibitor zDEVD-FMK. This tetrapeptide corresponds to the sequence at the cleavage site of PARP. During its proteolysis, zDEVD-FMK binds irreversibly to caspase-3, thereby inactivating the enzyme. As shown in Fig.5, increasing doses of zDEVD-FMK suppressed the apoptotic effect of butyrate. Complete inhibition of butyrate (10 mM)-induced apoptosis was observed with zDEVD-FMK concentrations of 100 μM and higher. However, when Caco-2 cells were treated with high butyrate doses (50-100 mM) this inhibition of apoptosis was only partial (69±6%). The caspase-inhibitor zVAD-FMK primarily inhibits caspases of the ICE-group, and at higher concentrations, also blockscaspases-3 and -6. ZVAD-FMK also significantly_reduced butyrate-induced Caco-2 cell apoptosis (Fig.5). No shift towards necrotic cell death in response to butyrate was observed when the caspase-cascade was completely blocked by high doses of zVAD-FMK (100 μM and higher).

Butyrate-induced upregulation of bak expression during Caco-2 cell apoptosis

Basal expression of the pro-apoptotic protein bak was low in Caco-2 cells. Expression of this protein was strongly upregulated by butyrate, in a dose-dependent manner (Fig.6a). Increased expression of bak in response to butyrate (50 mM) was detectable after 12-16h of butyrate treatment and progressively increased thereafter. Once again, in the presence of cycloheximide (100 μg/ml), butyrate induced bak-upregulation was completely blocked (Fig.6b). Caco-2 cell expression of the anti-apoptotic protein bcl-2 was not changed by butyrate, under all experimental conditions tested. Taken together, the data show that butyrate induced a strong shift in the ratio of anti- to pro-apoptotic bcl-family proteins, in favor of an apoptotic response.

DISCUSSION

This study provides new insight into the molecular mechanisms by which butyrate induces colon cancer cell apoptosis. Using the Caco-2 cell model, the data presented demonstrate that butyrate directly activates the intracellular caspase cascade. This classical apoptosis pathway transduces the apoptotic signal of a great variety of stimuli, including TNFα, Fas ligand, and others (Nagata and Golstein 1995, Nagata 1997). One endpoint of this cascade is the activation of caspase-3, which rapidly cleaves several key substrates implicated in the execution of apoptotic cell death, including PARP, DFF-45, lamin A etc (Nagata 1997). The inactivation of PARP leads to a breakdown of the DNA-repair system, allowing its degradation (Patel et al. 1996). The importance of this step in butyrate-induced apoptosis was further underscored in the present study. Inactivation of caspase-3 with zDEVD-FMK, which irreversibly binds with a very high affinity to the catalytic site of this particular caspase, completely inhibited butyrate-induced Caco-2 cell apoptosis.
The use of the broad-range caspase-inhibitor zVAD-FMK, which blocks ICE-like caspases (caspase 1,4,5), at low concentrations and CPP-32-like caspases (caspase-3) at higher concentrations, confirmed the necessity of the caspase-cascade in the execution of butyrate induced apoptosis. Recent reports (Vercammen et al. 1998) indicated that complete caspase inhibition with this inhibitor may shift the apoptotic response to a necrotic form of cell death. This was demonstrated for the FAS ligand in murine L929 fibrosarcoma cells (Vercammen et al. 1998) and for TNFα in intestinal epithelial cells (Ruemmele et al., manuscript submitted). In contrast to these observations, no necrotic cell death was observed in Caco-2 cells in response to butyrate, despite complete caspase inhibition with high zVAD-FMK doses. The data indicate that distinct signaling mechanisms exist for butyrate, clearly different from those employed by the death-receptors FAS or the p55 TNF-R. Taken together, our results demonstrate that butyrate-induced Caco-2 cell apoptosis is caspase-3 dependent, in keeping with a recent report in other cell lines (Medina et al. 1997).
Butyrate is known to affect the expression of several genes potentially involved into the regulation of cell death (Young and Gibson 1995). It is well established that butyrate exerts many effects via the inhibition of histone deacetylase, which leads to chromatin relaxation, altering gene expression (Boffa et al. 1978).Our data led us to speculate that one of these newly transcripted gene products is responsible for the activation of the caspase cascade. The most likely candidate in the Caco-2 cell model might be one member of the bcl-2 gene family. These proteins are important regulators and modulators of apoptotic signaling, primarily acting at the mitochondrial level (Nunez and Clark 1994). Bcl-2 and bcl-xl are

potent inhibitors of the caspase-cascade, whereas other proteins of this family, such as bax or bak, possess potent pro-apoptotic activity (Miyashita and Reed 1995). Clem et al (1998) recently showed that bcl-xl directly interacts with specific caspases. Upon the induction of apoptosis, this protein is cleaved by caspases-1 and -3, thereby allowing further propagation of the initial apoptotic signal. Our data indicate that the butyrate-induced pathway is closely involved with the mitochondrial bcl-pathway, known to be critical to apoptosis in other models (Ito et al. 1993, Oltavi et al. 1993). In this study, butyrate markedly upregulated the expression of pro-apoptotic bak, without altering Caco-2 cell bcl-2 expression. Time-course analysis of this upregulation revealed that the increased bak-expression was detectable just prior or concomitant to the occurrence of Caco-2 cell apoptosis, after 12-16h of butyrate stimulation. Previous reports (Moss et al. 1996) clearly delineated that bak expression correlated positively with apoptosis in the normal intestinal epithelium. Furthermore, colon cancer cells, significantly more resistant to apoptosis than non-transformed colonocytes, display reduced levels of bak, whereas the expression of other pro-apoptotic bcl-2 family members, such as bax, is unaltered (Krajewski et al. 1996). The ratio of pro- to anti-apoptotic bcl-2 proteins is an important regulator of a cell's susceptibility to undergo apoptosis upon a specific pro-apoptotic stimulus. Therefore, our observation of an increased bak expression in butyrate-stimulated Caco-2 cells points out to an additional, important effect of this particular short chain fatty acid by which it can enhance a colon cancer cell's susceptibility to apoptotic stimuli.

The relatively long lag-interval (14-16h) between butyrate-stimulation and the induction of apoptosis is very suggestive of additional intermediate steps requiring protein synthesis. Indeed, inhibition of protein-synthesis with cycloheximide completely abolished butyrate induced Caco-2 cell apoptosis, in keeping with reports in other cells (Medina et al. 1997). Furthermore, no butyrate induced activation of caspase-3 was observed in the presence of cycloheximide, suggesting that protein synthesis was required upstream of the caspase-cascade. To test the hypothesis that butyrate-induced bak upregulation as a critical step requires protein synthesis, the expression of this pro-apoptotic bcl-2 family member was analyzed at various time points in the presence or absence of cycloheximide. No butyrate induced upregulation of bak was observed when protein synthesis was completely blocked with cycloheximide, even after prolonged stimulation (36h). Inhibition of protein synthesis at various time intervals after the onset of stimulation with butyrate revealed that cycloheximide completely suppressed butyrate-induced apoptosis when added during the first 8h of butyrate stimulation, with progressively less effect when added later on. Addition of cycloheximide after 14h of butyrate-stimulation, a time point where bak is already upregulated, failed to reduce Caco-2 cell apoptosis rate, indicating that inhibition of

bak expression might play an important role in butyrate-induced Caco-2 cell apoptosis. However, we cannot completely exclude the possibility that additional events, upstream of bak ,which also require protein synthesis, are implicated.

The major sources of intestinal butyrate are dietary, as an important endproduct of the colonic bacterial fermentation of fiber or milk fat. Butyrate concentrations effective in inducing cancer cell apoptosis are easily achieved under physiological conditions in the colon. Normal, non-transformed colonocytes use these short chain fatty acids as a major energy source. However, as clearly demonstrated in this and other studies, colon cancer cells are rapidly induced to undergo apoptosis when incubated with butyrate (Hague et al. 1993, McIntyre et al. 1993). In summary, in this report we have uncovered two major pathways which mediate the apoptotic response to butyrate in colon cancer cells: the caspase-cascade and via upregulation of the pro-apoptotic protein bak at the mitochondrial level. Additional studies are needed to further elucidate the mechanisms upstream of caspase-3.

MATERIAL AND METHODS

Cell culture and materials: Caco-2 cells between passages 30-45 (American Type Culture Collection, Rockville, MD) were cultured at 37°C with 5% CO_2 in Minimal Essential Medium (MEM, Gibco, Grand Island, NY), containing 1% penicillin/streptomycin, 1% MEM non-essential amino acids (Gibco) and 5% fetal calf serum (FCS, Gibco). The agents used in this study were: Na-butyrate (Sigma, St. Louis, MO), the protein inhibitor cycloheximide (Sigma), the caspase-3 substrate and inhibitor zDEVD-FMK (Kamyia, Thousand Oaks, CA), the ICE-inhibitor zVAD-FMK (Kamyia), anti-caspase-3 antibody (Pharmingen, Mississauga, Ont.) which recognizes the native (32kDa) and the proteolytically active form (17kDa), anti-PARP-antibody (Stratagene), anti-mouse-horseradish peroxidase (Promega, Madison, WI), anti-bcl-2-FITC antibody (DAKO, Mississauga, Ont.), anti-bcl-2 antibody (Oncogene, Cambridge, MA), anti-bak-antibody (Pharmingen), propidium iodide (PI, Sigma), and HOECHST 33342-DNA stain (Boehringer, Mannheim, Germany).

Proliferation assay: These experiments were performed in 24-multi-well plates (Falcon Plastics, Oxnard, CA) at a density of $5x10^4$ cells/ml. After a 24h stabilization period in FCS-free medium, Caco-2 cells were cultured in the absence (control) or presence of butyrate (0.01-10 mM). Proliferation was quantified by monitoring changes in DNA synthesis, as measured by ^{3}H-thymidine uptake (2µCi/ml) added during the last 2 h of culture, as previously described (Ruemmele et al. 1998). Results were expressed as percentage of ^{3}H-incorporation relative to control wells without butyrate.

Apoptosis assays: Caco-2 cell were cultured in 24-multi-well plates to subconfluency and stimulated with butyrate (0.1-100 mM) and/or zDEVD-FMK (0.1-200 µM), or zVAD-FMK (1-300 µM) for up to 72h. In parallel, experiments were performed in the presence of cycloheximide (100 µg/ml) added simultaneously or after various time intervals (3-24h) after butyrate-stimulation. Apoptotic cells were identified using the Apo-Alert Annexin V kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. This allows the detection of phosphatidyl-serine on the external cell membrane early in apoptotic cell death. Increased permeability for the DNA-dye PI is noted during late phase apoptosis. Butyrate-induced apoptosis was thereafter quantified by flow cytometry (FACScan, Becton Dickinson, Mississauga, Ont., Canada). In parallel, the typical morphological criteria of apoptosis were confirmed by immunofluorescence after staining with the DNA-dye HOECHST 33342 (1µg/ml).

Immunoblotting: The activation of caspase-3 and subsequent degradation of poly-(ADP-ribose) polymerase (PARP) were determined by western blotting. In addition, changes in the expression of bcl-2 or bak were monitored by immunoblotting. Butyrate (0.1-100mM)- or butyrate+ cycloheximide(100 mg/ml)-treated and control Caco-2 cell lysates were prepared using an ice-cold lysis buffer (50mM Tris, 150mM NaCl, 10 mM EDTA, 1% Triton) and a mixture of protease inhibitors (Boehringer). After determination of protein concentrations, equivalent samples were resolved on 8%-14% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). For immunodetection, the membranes were incubated overnight with anti-caspase-3- (1:1000), anti-PARP- (1:3000), anti-bcl-2 (1:100) or anti-bak-antibody (1:800) in Tris-buffered saline/Tween-20-1% milk powder, followed by incubation with the corresponding alkaline phosphatase-conjugated antibody (anti-mouse-IgG 1:2000, anti-rabbit-IgG 1:2500). The bands were read by enhanced chemiluminescence (ECL-kit, Amersham).

Experimental design and statistical analysis: All experiments were performed in duplicate and were repeated at least four times. Representative experiments or mean values ± standard deviation are shown. Statistical significance was determined by the Mann-Whitney U-test. Differences with p values < 0.05 were considered significant

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FIGURE LEGENDS

Fig.1 **Effect of butyrate on Caco-2 cell proliferation**. Caco-2 cells were cultured in the absence (control) or presence of butyrate at 0.01-10 mM for 20 h. Proliferation of the cells was measured by the incorporation of ^{3}H-labeled thymidine, added 2 h prior to termination of the experiment. Basal proliferation without butyrate addition was set at 100%. Results are expressed as mean ± standard deviation versus control for 5 experiments (in triplicate).*p<0.01.

Fig.2 **Effect of butyrate on Caco-2 cell apoptosis**. a) Stimulation with butyrate 0.01-100 mM induced Caco-2 cell apoptosis in a dose-dependent manner as quantified by flow cytometry. *p<0.01. b) Immunfluorescence microscopy after staining with the HOECHST 33342 DNA dye revealed the typical features of apoptotic cells, with nuclear shrinkage, condensation and fragmentation in butyrate-treated Caco-2 cells. The nuclei of untreated cells have a uniformly regular, oval shape. Original magnification x800. c) Detection of the expression of phosphatidyl serine on the outer leaflet of the plasma membrane on apoptotic Caco-2 cells. During early apoptosis, butyrate-treated Caco-2 cells expressed phosphatidyl serine which binds FITC -labeled Annexin V, allowing quantification by flow cytometry. One representative of five similar experiments is shown.

Fig. 3 **Effect of protein synthesis inhibition on butyrate-induced Caco-2 cell apoptosis.** a) Simultaneous stimulation of Caco-2 cells with butyrate (20 mM) and the protein synthesis inhibitor cycloheximide (1-100 µg/ml), resulted in a clear suppression of butyrate induced apoptosis. b) Time course analysis after the addition of cycloheximide to butyrate-stimulated Caco-2 cells revealed that complete inhibition was observed within 8h, with progressively less protective effect thereafter. No significant protection was seen when cycloheximide was added 14h or later after butyrate-stimulation. *p<0.01

Fig.4 **Western blot analysis of the effect of butyrate on the caspase-cascade**. Immunoblotting was performed as described in Material and Methods. a) Butyrate (0.1-100 mM, 24 h) induced the cleavage of caspase-3 (32 kDa) into its active form (17 kDa). b) The activation of caspase-3 by butyrate (0.1-100 mM) was completely inhibited in the presence of the protein synthesis inhibitor cycloheximide (100 µg/ml). c) The caspase- 3 mediated degradation of PARP (113 kDa) into a 89 kDa fragment was clearly butyrate dose-dependent.

Fig. 5 **Inhibition of butyrate-induced Caco-2 cell apoptosis by zDEVD-FMK**. Butyrate-induced Caco-2 cell apoptosis was quantified by flow cytometry

using the Apo-Alert-Annexin V assay. The addition of zDEVD-FMK (0.1-300 μM), a caspase-3 inhibitor, potently blocked butyrate-induced apoptosis, in a dose-dependent manner. Similarly, the ICE-inhibitor zVAD-FMK, a broad-range caspase inhibitor, blocked butyrate induced Caco-2 cell apoptosis in a dose-dependent manner, without shifting the apoptotic response to a necrotic form of cell death. *$p<0.01$

Fig.6 **Effect of butyrate on Caco-2 cell bcl-2/bak protein expression.** a) Western blot analysis revealed that butyrate did not change Caco-2 cell bcl-2 expression, whereas bak was strongly induced by butyrate (0.1-100 mM, 24h). b) Time-course analysis revealed an increased bak expression after 12-16h in response to butyrate (50mM). This upregulation was completely suppressed in the presence of cycloheximide (100 μg/ml).

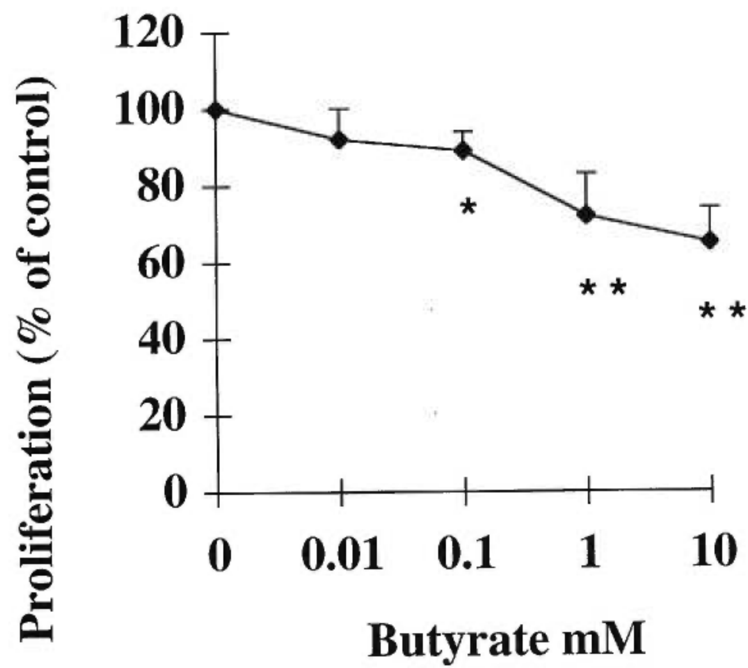
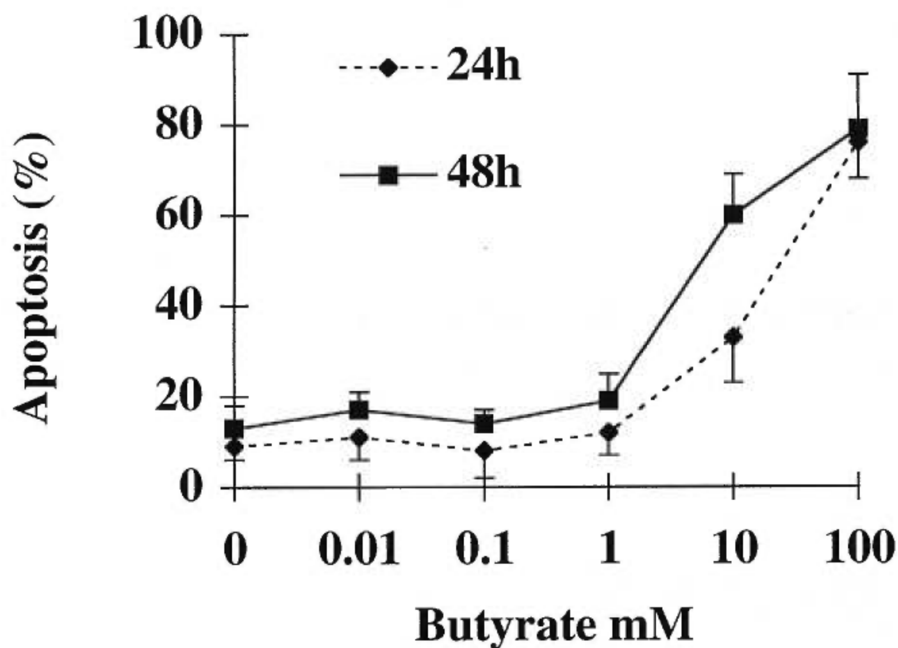


Fig.1



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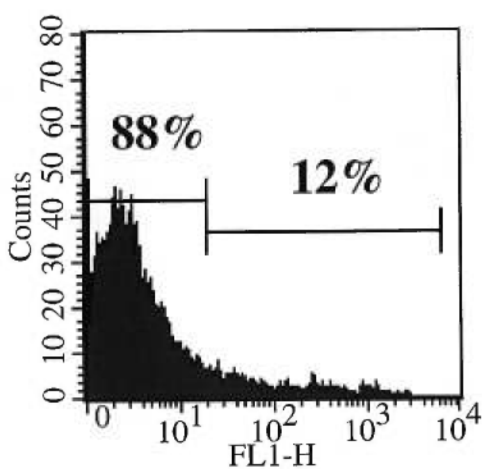

BUTYRATE 10 mM

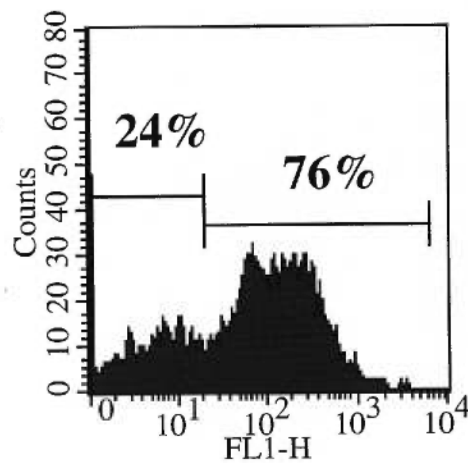

Fig.2a,c

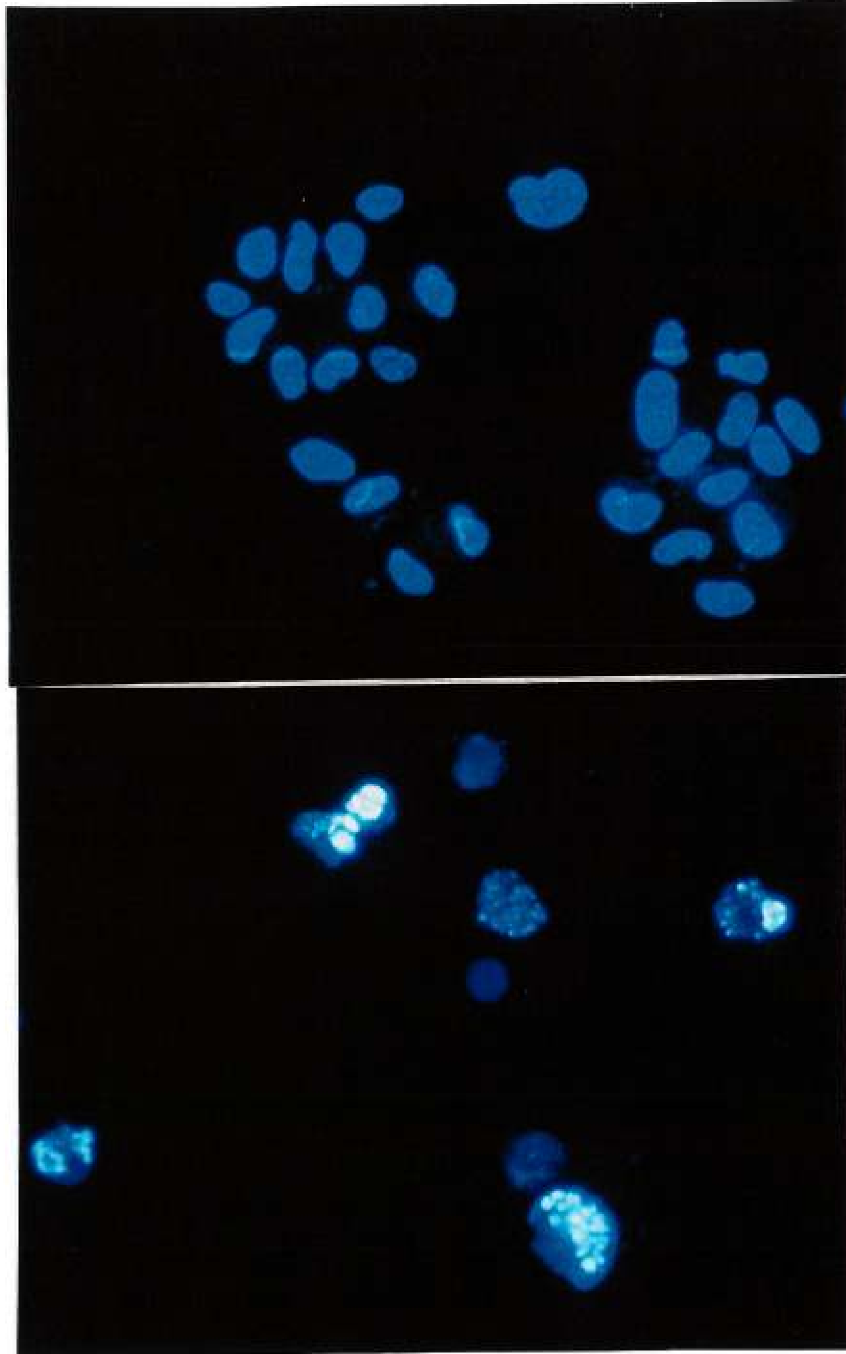

Fig.2b

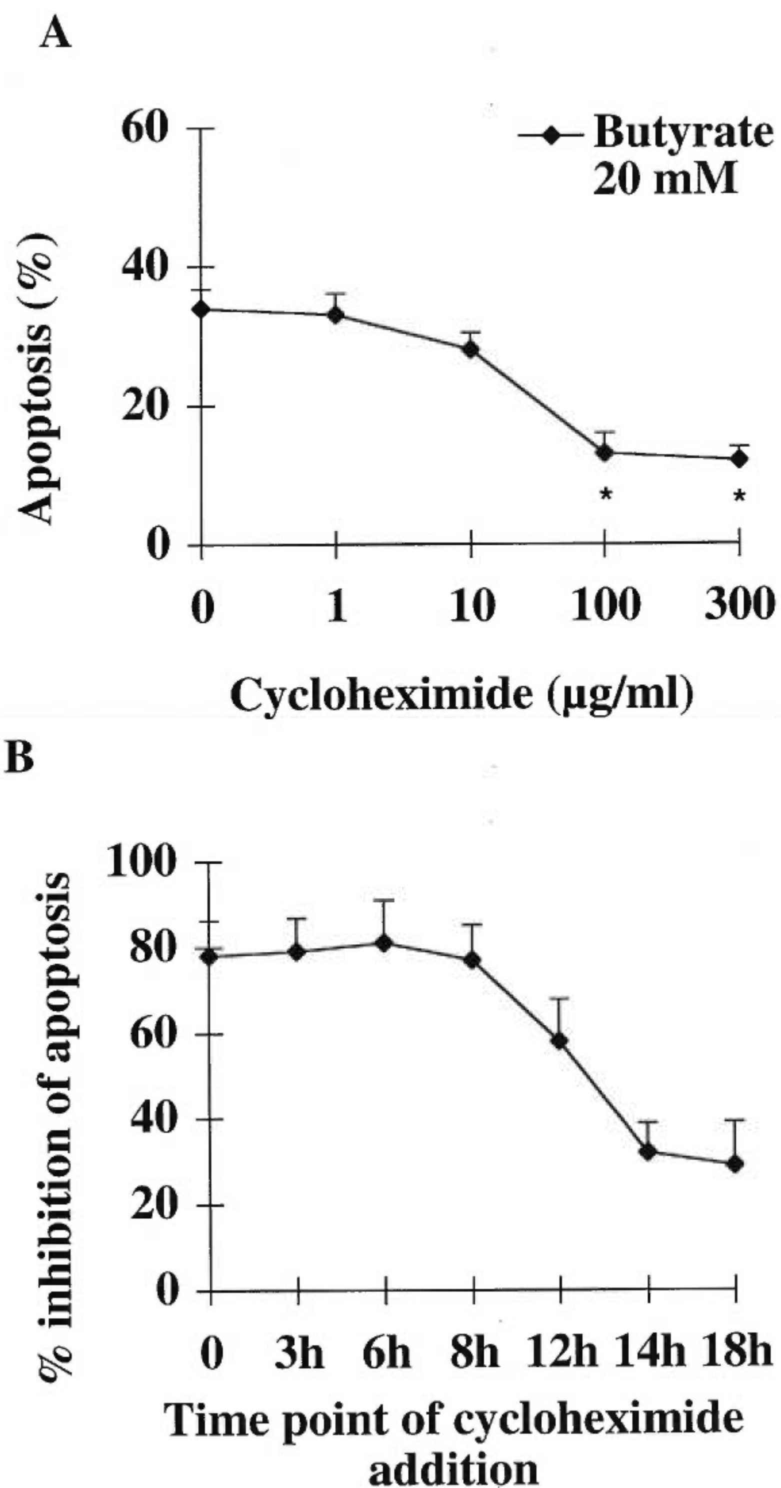

Fig.3

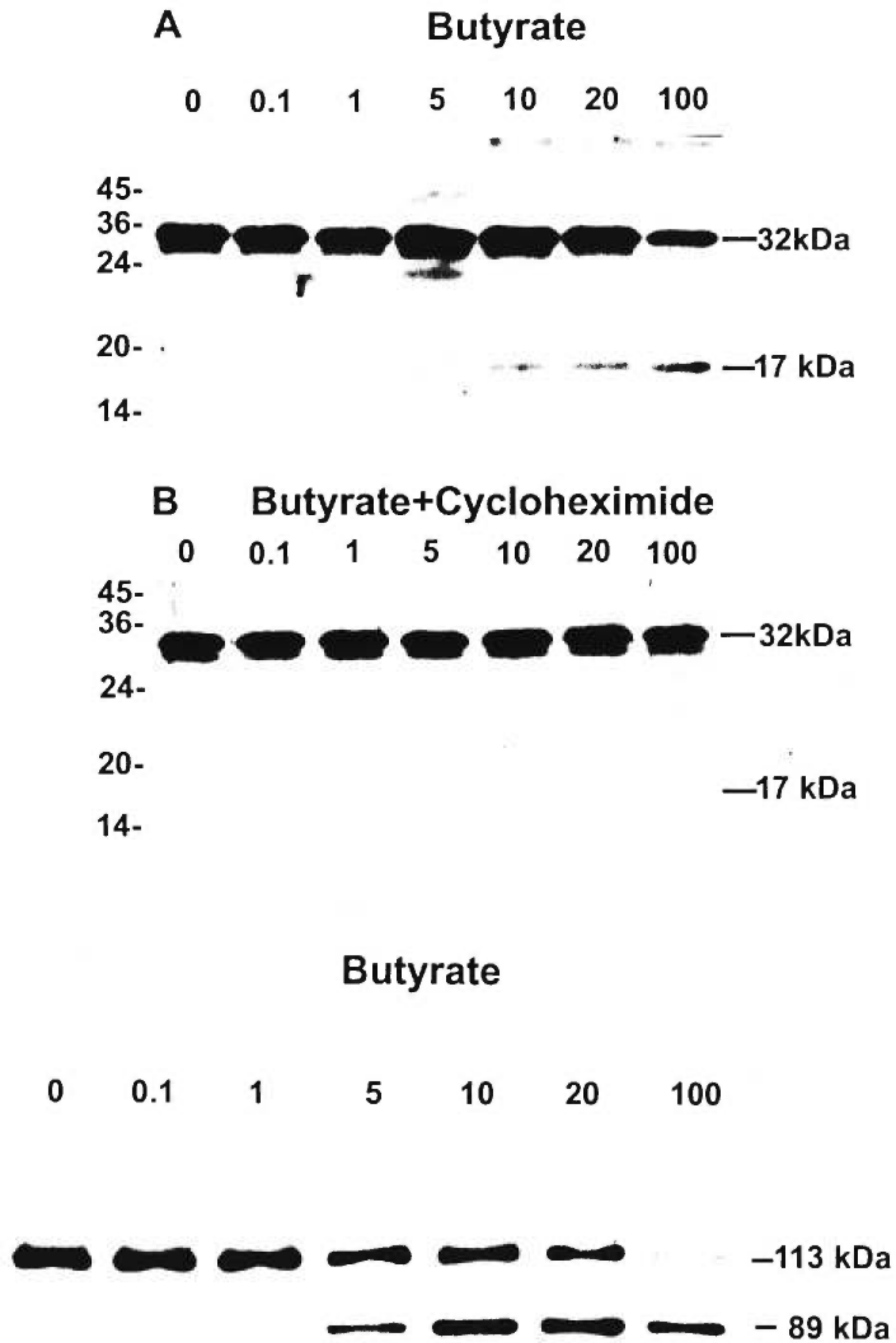

Fig.4

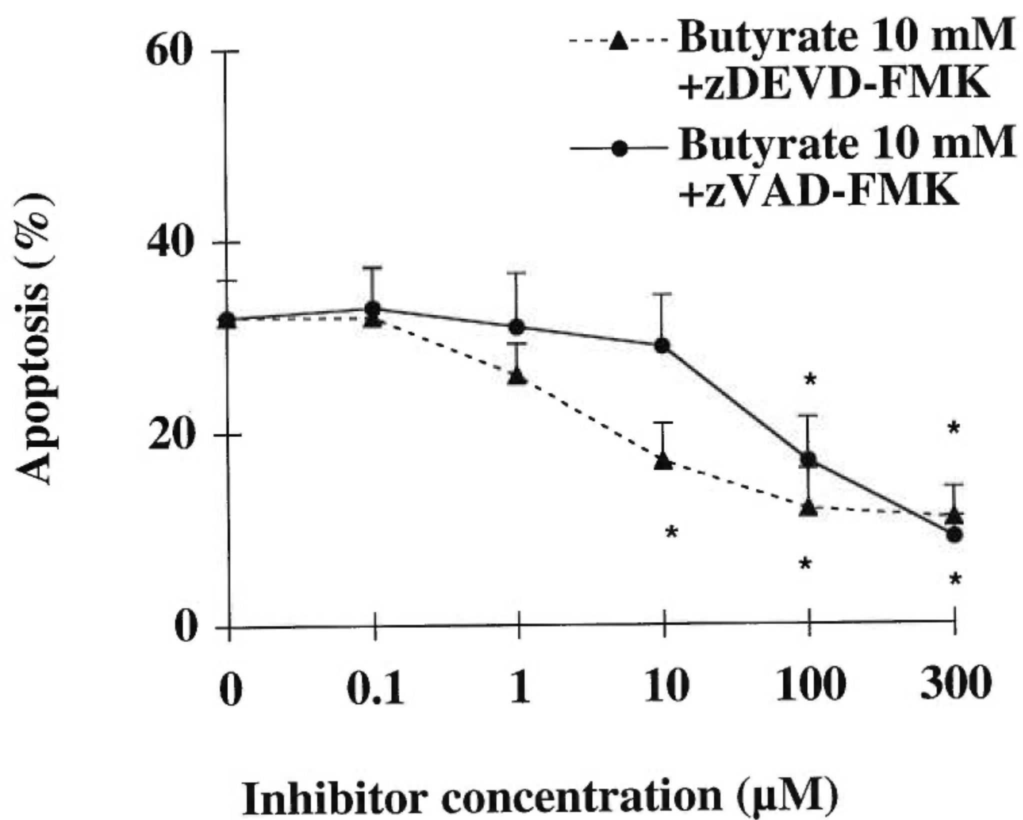

Fig.5

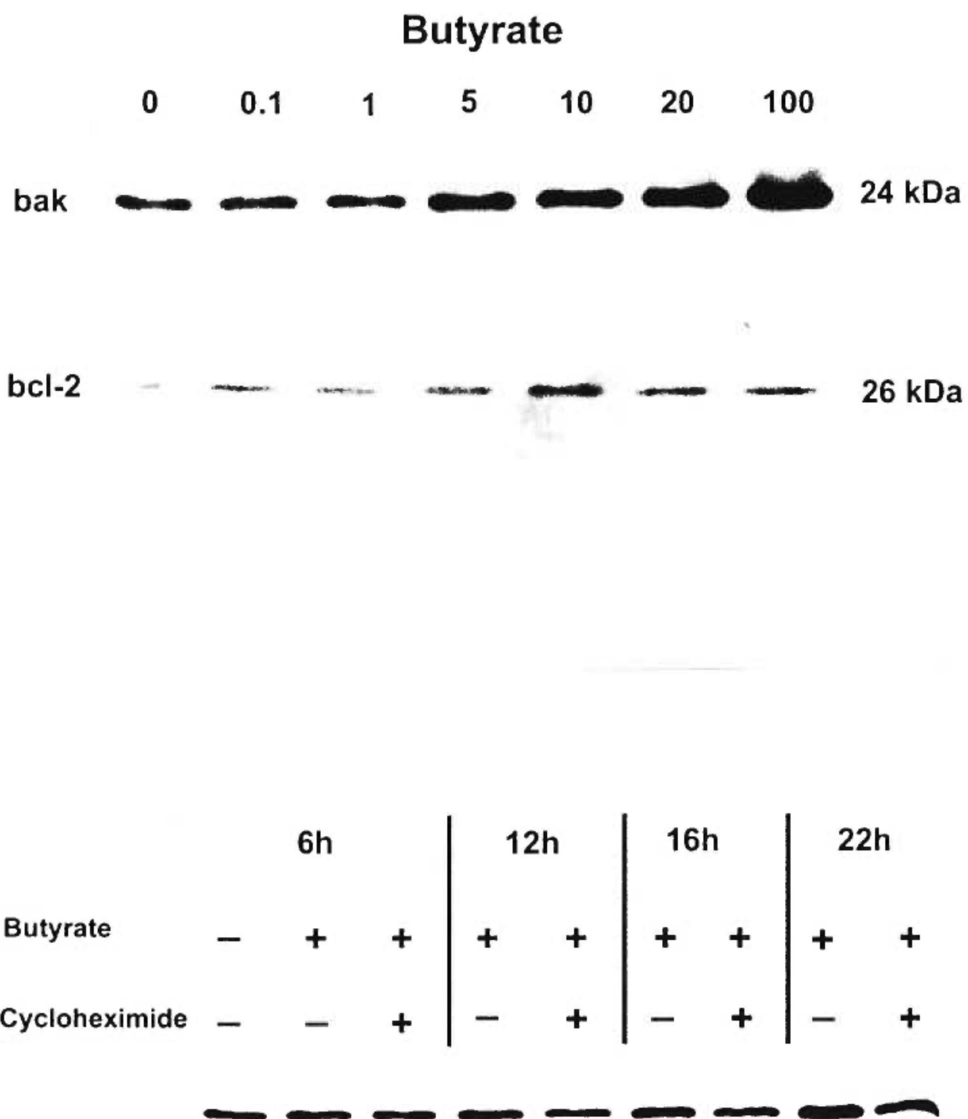

Fig.6

IV. DISCUSSION

There is increasing evidence that cytokines and certain growth factors play an important role in regulating homeostatic intestinal epithelial cell turnover. Alterations in the production of these regulatory factors, as seen in chronic idiopathic inflammatory bowel disorders, are associated with marked changes in intestinal epithelial cell turnover. The major aim of this study was to uncover the mechanisms by which pro-inflammatory cytokines result in pathological enterocyte turnover, with special emphasis on apoptosis. In a first step, we identified IFNγ and TNFα as potent inhibitors and stimulators of immature crypt cell growth, respectively. Using the IEC-6 cell model, TNFα-induced proliferation was mediated by the MAP kinase signalling cascade, as seen with EGF. IFNγ arrested immature crypt cells in the quiescent phase of their cell cycle, completely inhibiting enterocyte cell growth. A clear signalling hierarchy was observed between these two cytokines, with the anti-proliferative effect of IFNγ predominating. Depending on cytokine concentration and TNF-R stimulated, TNFα played a dual cytotrophic and cytotoxic role, as seen as enterocyte growth promotion or induction of apoptosis. Further analysis of the signalling mechanisms underlying TNFα's effects on IEC-6 cells revealed that ICE-caspases were critical to trigger apoptosis, whereas caspase-3 was not involved. In contrast, butyrate-induced Caco-2 cell apoptosis was strongly dependent on this particular protease, indicating distinct signalling pathways upon specific pro-apoptotic stimuli in these cell lines. Furthermore, complete inhibition of the caspase-cascade resulted in necrotic IEC-6 cell death after stimulation with TNFα. These findings suggest that the apoptosis pathway might play an important protective role against necrotic cell death. In addition, the pro-inflammatory cytokines IFNγ and TNFα also modulate apoptosis via the FAS pathway. Both cytokines, individually and in combination, markedly increased the susceptibility of enterocytes to FAS-induced apoptosis. Among important underlying mechanisms was the upregulation of enterocyte FAS-expression. The observed pro-inflammatory cytokines' effects were not limited to the regulation of intestinal epithelial cell proliferation and apoptosis. Cross-talk between immune and intestinal epithelial cells was also involved. This is particularly important since the intestinal mucosa is

chronically exposed to various antigens, microbial products and toxic factors present in the intestinal lumen. In this study, we analyzed the regulation of enterocytic MHC class II expression by pro-inflammatory cytokines, as an example of enterocyte-immune cell interactions. IFNγ and TNFα were potent upregulators of immature crypt cell class II expression. This potentially results in altered antigen-presentation by enterocytes, a particularly important component governing mucosal immune responses. Taken together, our data provide clear evidence, that pro-inflammatory cytokines such as IFNγ and TNFα act directly on intestinal epithelial cells. Both factors are, at the same time, implicated in regulating intestinal epithelial cell turnover, and serve as important mediators of intercellular cross-talk in the intestinal mucosa. Based on our findings, it is evident that alterations in the concentration of these pluripotent cytokine can lead to deleterious changes in the closely regulated intestinal epithelial cell turnover and consequently, intestinal functions.

Aberrant pro-inflammatory cytokine production in IBD induces altered intestinal epithelial cell turnover

Cytokines and growth factors are important regulators of normal intestinal epithelial cell turnover (Podolsky 1993, Ruemmele and Seidman 1998). The delicate balance between factors with growth promoting or inhibitory properties as well as anti- or pro-apoptotic effects is crucial to normal function and morphology of the intestine. The intestinal mucosal immune system constitutes an important source of cytokines and growth factors. The close proximity between epithelial cells and immune cells, especially intraepithelial lymphocytes allows a very intensive bi-directional exchange for intercellular "cross-talk" (Evans et al. 1992, Ferguson 1977, Kohyama et al. 1997). In fact, recent reports confirmed the necessity of normal mucosal T-cell function and cytokine production for physiological growth and maturation of the intestine (Thompson et al. 1996). Significant alterations in local cytokine production and release have been documented in various inflammatory conditions such as Crohn's disease, ulcerative colitis or celiac disease (Braegger et al. 1992, Dionne et al. 1998, MacDonald et al. 1990). These changes are believed to cause major perturbations in the turnover rate of intestinal epithelial cells, manifested by increased cell loss via apoptosis, along with

increased proliferation and crypt cell hyperplasia (Mac Donald and Spencer 1988). The net result of these alterations is villus atrophy and a paucity of mature absorptive cells, leading to diarrhea (Leichtner et al. 1996 a,b).

Recently, with the help of several animal models, evidence was put forward that cytokines, produced by activated resident or newly recruited immune competent cells are involved in the dysregulation of intestinal epithelial turnover (Guy-Grand et al. 1998, Benjamin et al. 1998). Activation of murine T-lymphocytes by the bacterial superantigen SEB, administrated intraperitoneally in normal mice, resulted in rapid onset of enteropathy. Morphological assessment of the intestinal mucosa after SEB-injection, revealed a time- and dose-dependent intestinal mucosal damage, characterized by reduced villus height and increased crypt depth (Benjamin et al. 1998). However, under the same experimental conditions, severe combined immune-deficiency (SCID) animals completely lacking T-cells, did not develop this enteropathy. On the other hand SCID-animals reconstituted with a CD4+ T-cell population showed the same pathological changes in the jejunum as normal wild-type animals (Benjamin et al. 1998). These experiments clearly demonstrate that activated immune-competent cells can directly alter normal intestinal epithelial cell turnover. Under physiological conditions, intraepithelial lymphocytes (IEL) play a particularly important role in maintaining homeostasis of intestinal epithelial cell turnover. In a mouse model, Boismenu and Havran (1994) showed that upon concavalin-A stimulation $\gamma\delta$+ IEL produced important amounts of keratinocyte growth factor (KGF), stimulating intestinal epithelial cell proliferation .

The imbalance between pro- and anti-inflammatory factors plays an essential role in the pathogenesis of IBD (Sartor 1995). Increased intestinal mucosal expression and production of the proinflammatory cytokines IFNγ, TNFα, IL-1β and IL-6 as well as the chemokines IL-8, monocyte chemoattractant protein-1 (MCP-1) or RANTES were reported in Crohn's disease and ulcerative colitis (Mahida et al. 1989, Youngman et al. 1993, Murch et al. 1991, Braegger et al. 1992, Reinecker et al. 1993, Izzo et al. 1993, Reinecker et al. 1995, MacDermont 1998). TNFα and IL-1β levels in the inflamed tissue correlated positively with disease severity (Sartor 1995, Brandtzaeg et al. 1997, Dionne et al. 1997). These two mediators possess important regulatory potential in terms of

turnover of both immune and non-immune cells such as fibroblasts, epithelial and endothelial cells. They are closely involved in the regulation of adhesion molecule expression and production/secretion of chemoattractant factors, promoting the accumulation of inflammatory cells in the intestinal tissue (MacDermont 1998). Upon stimulation with TNFα or several bacterial products, intestinal epithelial cells themselves secrete important amounts of chemoattractant factors, such as IL-6, IL-8 etc., thereby enhancing the inflammatory process in a positive feedback loop (Jung et al. 1995, McGee et al. 1993, 1995). These findings indicate that intestinal epithelial cells are not simply innocent bystanders, passively injured by immune cells and their mediators. Rather, intensive interactions between intestinal epithelial and immune competent cells are critical to maintain and perpetuate the chronic inflammatory process characteristic of IBD (Ruemmele and Seidman 1998). However, the initial pathogenetic mechanisms as well as the events that trigger inflammatory relapses and the underlying mechanisms that result in dysregulation of the intestinal mucosal immune system remain unknown. To date, the cause of IBD is considered to be multifactorial, with contributions from a genetic predisposition, environmental, as well as immunological factors (Fiocchi 1998, Cohen et al. 1998).

There is increasing evidence that **TNFα** is a cytokine central to the pathogenesis of immune-mediated bowel disorders (Maeda et al. 1991). Recent reports of the successful induction of remission in treatment-resistant CD patients using chimeric anti-TNFα antibodies (Van Dullemen et al. 1995; Targan et al. 1997) further support this concept. In some cases, a single injection of anti-TNFα antibodies resulted in prompt clinical improvement with normalization of disease activity index scores. However, our understanding of the underlying molecular mechanisms as to how TNFα contributes to intestinal inflammation and to the damage of the intestinal epithelium, is very limited.

Pro-inflammatory cytokines and intestinal epithelial cell proliferation

In the present work, we first aimed to analyze the biological effects of pro-inflammatory cytokines, significantly elevated in the intestinal mucosa in zones of acute inflammation, on intestinal epithelial cell turnover. Given the importance of TNFα and

IFNγ in the pathogenesis of immune-mediated bowel disorders such as IBD and celiac disease, we chose to study these two pro-inflammatory mediators, individually and in combination. We employed the IEC-6 cell line for these experiments as a model of non-transformed immature crypt intestinal epithelial cells. Epithelial cells have been shown to possess receptors for IFNγ and TNFα as well as various other cytokines and growth factors (Ucer et al. 1985). Our findings in IEC-6 cells indicate that both of these cytokines exert potent effects on immature intestinal epithelial cell growth. Whereas TNFα is a potent promitogenic factor, at doses in the picomolar range, we observed a marked antiproliferative effect of IFNγ, in agreement with previous observations using ileal (Cerf-Bensussan et al. 1984) and carcinoma-derived colonic cell lines (Deem et al. 1991). This growth-inhibitory effect of IFNγ resulted from an arrest of IEC-6 cells in the quiescent G_0/G_1 phase of the cell cycle, completely blocking their exit into the active phases. Kominsky et al. (1998) recently suggested that this growth inhibitory IFNγ effect is mediated via specific CKIs. In an in vitro model of glioblastoma, IFNγ increased the levels of p21 leading to cell cycle arrest in the late G_1 phase. P21 is known to bind and thereby inhibit specific cyclin-cdk complexes, needed in the transition from the G_1 to S-phase of the cell cycle. (I. *Molecular mechanisms of cell cycle progression*). In the glioblastoma cell model a clear correlation between IFNγ –induced p21 expression and reduced cyclin-cdk2 activity was observed further explaining this observation.

On the other hand, no direct cytotoxic effect of IFNγ on intestinal epithelial cells was noted, even at high doses. This is an important finding, since IFNγ has also been shown to impair intestinal epithelial cell barrier function (Hollander et al. 1986). Electrophysiological studies indicated that this latter effect was mainly due to an alteration of tight junctions, critical for an intact epithelial barrier, rather than secondary to the disruption of this barrier due to cell death (Madara and Stafford 1989, Hiribarren 1993). In fact, increased intestinal permeability is a feature of active inflammation, and has been implicated as playing a role in the pathogenesis of various immune-mediated bowel disorders (Fiocchi 1998, Dionne et al. 1998). We hypothesize that IFNγ is critically involved in this pathological process.

In marked contrast to IFNγ, TNFα proved to be a strong stimulator of IEC-6 cell proliferation. Previous reports in the literature, based on either tumor-derived

colonocytes (Deem et al. 1991, Abreu-Martin et al. 1995) or transfected epithelial cells of mouse small bowel origin (Kaiser and Polk 1997) have noted different findings. Kaiser and Polk (1997) recently reported that depending on dose and the TNF-R stimulated, TNFα can exert both growth stimulatory or inhibitory effects in murine YAMC cells. In their model, the p75 TNF-R generated a pro-mitogenic response, whereas the p55 TNF-R was implicated in growth inhibition. Receptor-studies in our laboratory, revealed that the IEC-6 cell line (as well as HIEC, data not shown) express both the p55 and p75 TNF-R. However, TNFα induced IEC-6 cell growth was clearly mediated via the p55 TNF-R, as demonstrated by selectively stimulating this receptor with hTNFα or agonistic antibodies. The signalling pathways implicated in TNFα-induced growth stimulation of intestinal epithelial cells were unknown. Detailed analysis of the molecular mechanisms in our *in vitro* model using IEC-6 cells revealed that p55 TNF-R-mediated growth stimulation was dependent on the activation of the MAPkinases Erk1 and 2. PD 98059, a specific inhibitor of MKK, the kinase responsible for the dual-phosphorylation of Erk1/2 (Matsuda et al. 1992, Seger and Krebs 1995), significantly reduced basal as well as TNFα or EGF induced IEC-6 cell proliferation. The importance of this cascade in TNFα-stimulated enterocyte growth was confirmed by experiments using the MAPK assay, showing an increased phosphotyrosine-activity. In our experiments, we observed an early activation of Erk1/2 in response to TNFα which declined after 15-30 min., as demonstrated (Dionne et al. 1998). However, prolonged stimulation (1h or longer) of IEC-6 cells with TNFα, revealed a second peak after 1h (unpublished observations). This biphasic activation of the MAPkinases in IEC-6 cells after TNFα-stimulation is in concert with the observation of Meloche et al. (1992). It was speculated that the persistent phase of MAPKinase activation is essential to pass a restriction point of the cell cycle (Lenormand et al. 1993). Subsequent to stimulation and phosphorylation, the p42 and p44 MAPkinases are translocated into the nucleus (Lenormand et al. 1993). This rapid translocation is a prerequisite for Erk1/2 to exert their regulatory effects on cell growth via interaction with various transcription factors, implicated in the regulation of cell cycle progression (Angel and Karin 1991, Seger and Krebs 1995). For example, cyclin D1, essential to the progression from the G_0 to G_1-phase of the cell cycle and subsequently to the phosphorylation of the retinoblastoma tumor suppressor gene product (see I. *Molecular mechanisms of cell cycle progression*)

is one such product which is under the positive control of Erk1/2 (Lavoie et al. 1996). Activation of the MAPKinase cascade by expression of a constitutively active MKK1 in fibroblasts, significantly increased cyclin D1 gene expression and protein levels (Lavoie et al. 1996). Other examples are the transcription factor Elk-1, implicated in the induction of early genes containing a serum responsive element, and the nuclear protein c-fos which is rapidly phosphorylated by the MAPkinases in various *in vitro* models (Seger and Krebs 1995). Subsequently, phosphorylated c-fos, as well as other nuclear proteins such as fosB or fra-1 together with activated c-jun, junB or junD form the transcription factor AP-1 (Ransone and Verma 1990). It was shown that mRNA levels of c-fos and c-jun are markedly elevated in rapidly proliferating 3T3 cells, in contrast to highly differentiated cells, indicating their involvement in the regulation of cell growth (Greenberg et al. 1984). In contrast to the activation of c-fos via MAPkinases, phosphorylation can also inactivate this transcription factor in some models. Chen et al. (1993) described that ribosomal protein S6 kinase (RSK) can directly enter the nucleus and phosphorylate c-fos on serine 362, adjacent to the specific site of Erk1/2 (serine 374). After dual-phosphorylation however, this transcription factor is markedly inhibited. It is important to note that RSK is a substrate of Erk1/2, which regulate RSK activity by specific phosphorylation (Nguyen et al. 1990). These findings indicate a dual role of Erk1/2 in the regulation of AP-1, potentially leading to either its activation or inhibition.

The observation that total inhibition of the MAPkinase kinase with PD98059 did not completely block the promitogenic effect of TNF suggests that this particular cytokine activates other signalling pathways. Extending our original findings with TNF, we observed very recently that the SAPkinase pathway is also implicated in p55 TNF-R mediated IEC-6 cell growth (manuscript in preparation). Both SAPkinase pathways, p38 and p46-p55-JNK, were rapidly activated in response to TNFα, with a peak after 10 min. These findings, based on specific phosphorylation observed using western blots, were confirmed by kinase assays using c-jun fusion protein as substrate (Dionne et al. 1998). Furthermore, direct inhibition of p38 with SB203580 markedly reduced the promitogenic effect of TNFα, further supporting the hypothesis that the SAPK p38-pathway is necessary for TNF-induced IEC-6 cell growth stimulation. However, this

inhibitor is also known to interfere with the generation and the metabolism of specific prostaglandines. Borsch-Haubold et al. (1998) recently showed that this preferential inhibitor of p38 blocks also the activity of the cyclooxygenases (COX)-1 and 2 . This is of particular importance since prostaglandins might be implicated in the regulation of cell proliferation. On the other hand, the key enzyme of the prostaglandin pathway, cytosolic phospholipase A2, is also a known substrate of the MAPKinases. Further complicating is the observation that SB203580 inhibits c-raf in vitro (Hall-Jackson et al. 1999), whereas in vivo, a paradoxical activation of c-raf occurs in response to SB203580 without any increase in the GTP-loading of upstream ras. Taken together, these recent reports indicate the complexity and crosslinks between the different signalling pathways triggering intracellular events in response to specific cytokines and growth factors.

Our understanding of the intracellular signalling processes in response to TNFα has been enhanced by the discovery of numerous new adapter proteins which can be recruited to the p55 and p75 TNF-R (Nagata 1997, Ashkenazi and Dixit 1998)). Recently, it was proposed that TRAF-2 binds directly or indirectly via RIP to TRADD, which is closely linked to the p55 TNF-R trimer (Hsu et al. 1996, Liu et al. 1996). TRAF-2 is known to subsequently stimulate the MAPkinase and SAPkinase cascade (Liu et al. 1996). However, the MKKs upstream from Erk1/2, do not bind directly to TRAF-2 (Liu et al. 1996), suggesting the presence of another TRAF-2 binding kinase upstream of MKK-1. On the other hand, both TRAF-1 and TRAF-2 are classical adapter molecules to the p75 TNF-R (Rothe et al. 1994). These findings may at least partially explain why the two TNF-R can assume similar roles in regulating intestinal epithelial cell growth. If TRAF-2 is predominantly recruited to the p75 TNF-R this receptor would activate downstream MAP kinases and/or SAP kinases upon stimulation, thereby inducing enterocyte proliferation. On the other hand, if this adapter is mainly bound via TRADD or RIP to the p55 TNF-R, proliferation will occur in response to TNFα-induced oligomerization of the p55 TNF-R, as observed in the IEC-6 cell model.

Obviously, in the *in vivo* situation, isolated changes in the production and secretion of one particular cytokine or growth factor are rather uncommon. Most often, an entire cytokine profile changes, eg. from anti- to pro-inflammatory and vice versa. In

IBD tissue, a whole array of pro-inflammatory cytokines is produced in high concentrations (Sartor 1995, Fiocchi 1998). Therefore, we also analyzed the effect of the combination of TNFα and IFNγ on intestinal epithelial cell growth, using the IEC-6 cell model. Interestingly, we observed a clear hierarchy effect of IFNγ over TNFα. TNFα-induced growth stimulation was completely suppressed by IFNγ, even at minimal doses. The number of cells arrested in the quiescent phases of the cell cycle was not different if IFNγ was added alone or in the presence of TNFα. On the other hand, TNFα alone increased the number of cells entering the active phases of the cell cycle more then 5-fold. The signalling pathway employed by IFNγ in IEC-6 cells is also markedly different from those activated in response to TNFα (discussed below).

Beyond the two factors studied, other pro-inflammatory cytokines are known to interfere with intestinal epithelial cell growth. Experiments with the IEC-6 cell line indicate that interleukin (IL)-2 is a strong inhibitor of proliferation (unpublished data). Furthermore, IL-17, a recently discovered pro-inflammatory factor, potently inhibited growth of mouse enterocytes *in vitro*. Analysis of the underlying molecular signalling mechanisms indicate that the MAPkinases Erk1/2 and JNK are essential for this effect (Reinecker et al. 1998). Human intestinal epithelial cells are known to express receptors for various other cytokines, such as IL-2, IL-4, IL-7 or IL-9, which trigger a rapid tyrosine-phosphorylation on various intracellular proteins upon specific stimulation (Reinecker and Podolsky 1995). All these four receptors share the common γc chain, characteristic for the IL-2 receptor β, implicated in regulating various T- and B-cell functions. The significance of these interesting *in vitro* observations remains to be established.

Pro-inflammatory cytokines and intestinal epithelial cell apoptosis

Cytokines play a pivotal role as regulators of apoptosis in many different cell models. Given the dramatically increased rate of apoptotic cells seen in inflammatory conditions, we were particularly interested in deciphering the mechanisms as to how cytokines induce intestinal epithelial cell apoptosis. It is well established that the p55 TNF-R can trigger apoptosis in target cells (Nagata 1997). Over the last few years, a whole family of receptors (TNF-R superfamily) was described, characterized by a homologous extracellular sequence of cysteine rich repeats (Ashkenazi and Dixit 1998). However, only few of these receptors are known to activate intracellular apoptosis pathways. Those mainly involved are the p55 TNF-R and FAS, also called Apo-1 or CD95, and more recent members DR3, DR4 and ATAR. All these receptors are characterized by a typical intracellular death domain (Ashkenazi and Dixit 1998). This specific sequence allows the binding of adapter proteins via their own death domain which can directly or indirectly activate the caspase-cascade (Nagata 1997, Green 1998). Stimulation of the p55 TNF-R in IEC-6 cells only induced apoptosis when extremly high cytokine concentrations were employed, whereas simultaneous stimulation of both TNF-R were highly effective in triggering apoptosis. In the past, there has been conflicting reports on the involvement of the p75 TNF-R to trigger apoptosis. Heller et al. (1990) observed a markedly enhanced apoptotic response of HeLa cells to TNFα when both the p55 and p75 TNF-R were stimulated, whereas activation of the p55 TNF-R alone failed to induce apoptosis. In order to explain these observations, two different theories were discussed. 1) The p75 TNF-R alone is sufficient to activate the intracellular apoptosis signalling pathway, or 2) the interaction of the p75 TNF-R with the p55 TNF-R is necessary to induce apoptosis. Ligand-passing mechanisms between the p55 and p75 TNF-R have been suggested to result in a more potent apoptotic response (Tartaglia et al. 1993). However, with the help of Hela transfectants expressing wild-type p75 TNF-R or deletion mutants, this ligand-passing mechanism was not confirmed. Rather, intracellular signalling mechanisms shared between the two receptors were thought to be responsible for this interaction (Weiss et al. 1997). However, Lin et al. (1997) demonstrated that isolated stimulation of the p75 TNF-R triggered apoptosis in response to TNFα in activated mouse T-cells, confirming the

hypothesis that, at least in some models and under certain conditions, stimulation of the p75 TNF-R alone is sufficient to induce apoptosis. Our data with IEC-6 cells largely support the concept of p75 TNF-R enhanced apoptosis, since over 100-fold higher doses were required if the p55 TNF-R alone was stimulated, compared to simultaneous activation of both receptors.

Examining the signalling mechanisms of TNFα-induced IEC-6 cell apoptosis, we observed that zVAD-fmk, an ICE-caspase inhibitor, potently suppressed TNFα induced apoptosis. However, the caspase-3 specific inhibitor zDEVD-fmk failed to do so. However, the pro-enzyme, inactive caspase-3 was expressed in IEC-6 cells. These peptide inhibitors correspond to the sequence at the cleavage site of the specific substrates of each caspase (Garcia-Calvo et al. 1998). During their proteolysis, the fluoromethylketone form of these peptide inhibitors binds irreversibly to its specific caspase, thereby inactivating this enzyme. Surprisingly, we observed a switch from TNFα induced apoptosis to a necrotic form of cell death when ICE-caspases were completely blocked by administration of high doses of zVAD-fmk. The inhibitor alone thus, was without cytotoxic effect. These findings indicate that several pathways are involved in TNFα-induced cell death in the IEC-6 cell model. If the apoptotic cell death pathway is completely blocked, the same stimulus employed a different signalling pathway resulting in necrosis. Our data are in keeping with recent reports showing that total block of ICE-caspases, intrinsically involved in the generation of FAS-mediated apoptosis, switched the apoptotic to a necrotic response in L929 cells (Vercammen et al. 1998 a,b). In contrast to that model, TNFα induced IEC-6 cell apoptosis was almost completely suppressed by low doses of zVAD-fmk. Only total caspase-inhibition employing higher inhibitor doses, led to a change into a necrotic form of cell death. We speculate, that zVAD-fmk doses of 50 μM and higher block other additional caspases essential to TNFα-induced apoptosis. Garcia-Calvo et al. (1998) recently showed that this inhibitor can block all caspases depending on the doses, supporting our hypothesis. It is very likely that zVAD-fmk blocks one or several of the responsible initiator caspases to trigger apoptosis in response to TNF. Therefore, in the presence of high doses zVAD-fmk, no activation of the caspase cascade occurs after stimulation with this

cytokine, allowing the propagation of otherwise suppressed signals leading to necrotic cell death.

Isolated stimulation of the p55 TNF-R did only induce IEC-6 cell apoptosis when extremly high cytokine concentrations were used. However, costimulation of these cells with IFNγ rendered them more susceptible to p55 TNF-R mediated apoptosis. This observation is in keeping with previous reports using HT-29 and other cells (Abreu-Martin et al. 1995, Ossina et al. 1997, Keane et al. 1996). The molecular mechanisms underlying this sensitizing effect of IFNγ to TNFα induced apoptosis remained unclear until very recently. In some models, IFNγ was shown to upregulate the expression of the p55 TNF-R. Using the HT-29 cell model, Ossina et al. (1997) provided evidence that IFNγ is a potent modulator of apoptosis-related gene expression. The increased sensitivity of HT-29 cells to TNFα as well as FAS ligand induced apoptosis after IFNγ pretreatment was preceded by an increased mRNA expression of several caspases of the ICE- (1,4) and CPP-32-group (caspase-3, -8). Furthermore, the pro-apoptotic bcl-2 family member bak was also induced by this particular cytokine. All these IFNγ-induced events can be considered as pro-apoptotic, in concert with the observation that IFNγ alone induced 20-30% apoptosis in HT-29 cells (Abreu-Martin et al. 1995, Ossina et al 1997). In our experiments using IEC-6 as well as HIEC lines, IFNγ did not induce apoptosis despite doses as high as 1000 U/ml. However, we observed a similar sensitizing effect of IFNγ to TNFα induced apoptosis in both cell lines. Therefore, we suspect that similar intracellular signalling mechanisms, as proposed for HT-29 cells, account for this phenomen in IEC-6 and HIEC cells.

Very recently, evidence was put forward that the classical IFN - Janus protein kinase (Jak) - signal transducers and activator of transcription (STAT) pathway, also participates in TNFα induced apoptosis (Kumar et al. 1997). It is well known that IFNγ binding leads to aggregation of its receptor with subsequent formation of a multicomplex, comprising two receptor-subunits and the recruited kinases Jak 1 or 2 (Darnell et al. 1994). Upon binding, these Jaks become activated and phosphorylate the receptor and themselves on a specific tyrosine residue. Signal transmission of the activated multicomplex is mediated via phosphorylation of specific cytoplasmic

substrates, including the STAT proteins (Ihle 1994). Phosphorylated STATs form homodimers which translocate to the nucleus and activate the transcription of specific genes containing GAS (gamma-activated sequence) elements (Ihle 1994). Kumar et al. (1997) recently demonstrated that TNFα failed to induce apoptosis in STAT1-null cells. Analysis of the underlying mechanisms revealed that these mutant human fibroblasts expressed markedly reduced amounts of caspases 1, 2 and 3 mRNA. However, the amounts of other caspases were similar to the expression in non-mutant paternal cells. Reintroduction of STAT1α in the mutant fibroblasts restored both the sensitivity to TNFα induced apoptosis and the expression of the deficient caspases. However, this restoration was independent of the signalling functions of STAT since variant STAT proteins that carried point mutations which inactivated domains required for the formation of homodimers were equally effective. Normal caspase mRNA levels and a normal sensitivity to TNFα induced apoptosis were observed. These data indicate a close relationship between the IFNγ and these intracellular apoptosis pathways also in non-tumoral cells. This is in keeping with our observation of IFNγ-enhanced susceptibility to TNFα-induced apoptosis in IEC-6 cell, which is a non-transformed and non p53-mutant cell line derived from mature rat intestinal epithelium (Quaroni et al. 1979).

FAS-FAS ligand interaction and intestinal epithelial cell apoptosis

Another important membrane receptor potentially implicated in enterocyte apoptosis is FAS (Strater et al. 1997, Sakai et al. 1997). Similar to the p55 TNF-R, this receptor is characterized by an intracellular death domain, which directly binds the adapter molecule FADD (Nagata 1997, Ashkenazi and Dixit 1998). To date, the FAS-pathway is the best characterized among apoptosis signal transduction pathways (Nagata 1997). Upon stimulation, this receptor forms a classical DISC, resulting in the rapid autolytically activation of caspase-8 (Boldin et al. 1996). In contrast to p55 TNF-R triggered apoptosis, TRADD is not involved in this process (Nagata 1997. Upon binding of the FAS ligand, FAS oligomerization allows direct binding of FADD via its DD to this complex. Furthermore, FADD possesses a death effector domain (DED) at its N-terminal region, responsible for the activation of caspase-8. This subsequently

stimulates other specific effector caspases. *In vitro* studies revealed that the potent effector protease caspase-3 is the preferred substrate of caspase-8 (Boldin et al. 1996). However, activation of ICE-like caspases in response to FAS engagement was also reported, indicating that the FAS-pathway is more complex than the above proposed model (Nagata 1997). Furthermore, FAS is also known to activate other intracellular pathways resulting in the production of pro-inflammatory factors, such as cytokines or chemokines (IL-8) (Abreu-Martin et al. 1995). It was suggested that the SAP kinase pathway resulsting in stimulation of activator protein-1 (AP-1) is implicated in this function (Abreu 1997).

Recently, Strater et al. (1997) reported that the FAS ligand-FAS mechanism plays an important role in colonocyte apoptosis and is thus potentially involved in the pathogenesis of ulcerative colitis. Colonocytes constitutively express FAS on their surface, as well as human enterocytes, as we demonstrated with HIEC cells. We also observed, using *ex vivo* colon explant cultures, a region dependent difference in the intensity of FAS expression, with a more intense signal on mature epithelial cells compared to immature crypt cells, in concert with other reports (Leithauser et al. 1993). Upon stimulation with an anti-FAS-IgM antibody, colonocytes rapidly underwent apoptosis. In contrast to the observation of a high sensitivity to FAS-induced apoptosis of isolated colonic crypts, FAS triggered apoptosis occurred only when very high agonistic antibody-doses were employed in our *ex vivo* explant culture model. This significant difference compared to the report of Strater et al. (1997) might be due to the different experimental conditions employed. For instance, Grossmann et al. (1998) uncovered that human intestinal epithelial cells rapidly undergo apoptosis after isolation, due to the loss of contact with the basal membrane. Therefore, the isolation of whole crypts might also facilitate apoptosis upon a specific trigger, such as the FAS ligand. On the other hand, explant cultures appear to be less sensitive, since the tissue's integrity is better preserved, similar to the situation *in vivo*. The response to FAS-stimulation in our model, however, was markedly increased in the presence of the pro-inflammatory cytokines IFNγ and/or TNFα. As shown by in situ TUNEL-staining, apoptotic cells were seen all along the crypts after costimulation with FAS ligand and IFNγ. One of the underlying mechanisms of this increased sensitivity to FAS-induced

apoptosis is cytokine-induced upregulation of FAS. Increased FAS expression was reported in the inflamed tissue of patients with IBD (Reich et al. 1996). Therefore, we speculate that IFNγ and/or TNFα, both abundantly secreted by the infiltrating immune-competent cells, mediate this effect, as we demonstrated in our *ex vivo* explant and *in vitro* cell model.

Our finding that HIEC are FAS-positive, yet completely resistant to the induction of apoptosis upon stimulation of this receptor, despite extremely high agonistic antibody-doses, was surprising. Similar to the situation observed in *ex vivo* colon cultures, the pro-inflammatory cytokines TNFα and IFNγ alone, and more potently in combination, rendered these intestinal epithelial cells highly susceptible to FAS-induced apoptosis. We found that this sensitizing effect is, at least in part, due to a specific upregulation of surface expression of FAS by these cytokines individually and synergistically to a great extent. Other mechanisms proposed to explain this phenomen were the upregulation of various pro-apoptotic gene products. Keane et al. (1996) demonstrated that IFNγ enhanced the susceptibility to FAS-induced apoptosis in breast epithelial cells via the selective increase of caspase 1 expression. In addition to an increased expression of caspases that are implicated in FAS-signalling, a switch in the ratio of pro- to anti-apoptotic Bcl-2 gene products was proposed to explain this cytokine effect (Koshiji et al. 1996, Ossina et al. 1997). In a cell line of human colon cancer origin (COLO 201), TNFα as well as IFNγ upregulated the expression of pro-apoptotic bax, and at the same time downregulated bcl-2, resulting in a markedly increased pro-apoptotic ratio (Koshiji et al. 1996). We anticipate that similar events contribute to cytokine-induced sensitivity to FAS-induced apoptosis in the HIEC model. Taken together. these data further support the concept of an important regulatory role of cytokines in normal intestinal epithelial cell turnover.

To address the question as to whether FAS-FAS ligand interaction is implicated in normal intestinal epithelial cell turnover, we assessed FAS ligand induction and regulation via cytokines on intestinal epithelial cells. In contrast to a recent report (Iwamoto et al. 1996), we did not observe FAS ligand expression on immature or mature colonocytes nor in HIEC. In keeping with our findings, Moller et al. (1996)

showed that the only FAS ligand positive cells in the normal gut epithelium are Paneth cells. The latter are absent in the distal colon, the tissue used in our study. In their report, Iwamoto et al. (1996) showed weak FAS ligand expression on colonocytes in normal tissue and an overall FAS ligand expression on intestinal epithelial cells in zones of acute inflammation in ulcerative colitis. Therefore, we speculated that pro-inflammatory cytokines contribute to the induction of FAS ligand. However, our experiments in both the *ex vivo* explant culture model as well as HIEC failed to induce detectable levels of FAS ligand with IFNγ, TNFα, TGFβ or IL-1β, alone or in various combinations. This might be due to different experimental conditions. Alternatively, factors others than those we tested might contribute to FAS ligand expression on intestinal epithelial cells. However, other reports in the literature confirm our findings that non-transformed intestinal epithelial cells do not express FAS ligand in the absence (Moller et al. 1996) or the presence of inflammation (Strater et al. 1997). Unlike both TNF-Rs which can be involved in apoptosis and the modulation of intestinal epithelial cell proliferation, our experiments with HIEC showed that FAS was not implicated in normal intestinal epithelial cell growth regulation. However, in other cell systems, including leukemic cells, stimulation of FAS markedly enhanced mitogenic activity (Mapara et al. 1993). We thus have no strong evidence to support the hypothesis that FAS ligand is directly implicated in homeostatic intestinal epithelial cell turnover regulation under physiological conditions. Under pathological inflammatory conditions, enterocytes rapidly die by FAS-induced apoptosis. Therefore, it is very likely that FAS-FAS ligand interaction plays a pathogenetic role in intestinal epithelial damage, as seen in IBD. In the presence of immune-mediated inflammatory conditions, a great number of FAS ligand positive cytotoxic T-cells are recruited to sites of inflammation. We hypothesize that the increased amounts of pro-inflammatory cytokines present in actively inflamed tissue are critical to FAS-upregulation and induction of various pro-apoptotic intracellular factors, as discussed above.

In contrast to normal intestinal epithelial cells, tumoral transformed cells acquire a high degree of resistance to FAS-induced apoptosis (O'Connell et al. 1997). Analysis of the underlying mechanisms revealed that FAS was either not expressed, was at very low levels, or that this receptor was functionally uncoupled in these cells (Ito et al. 1991,

O'Connell et al. 1997, Leithauser et al. 1993, Moller et al. 1994). Furthermore, in some cancers, the intracytoplasmic domain of FAS was found to be mutated, and therefore failed to bind adapter molecules essential to trigger an intracellular response upon stimulation (O'Connell et al. 1997). However, several other factors confer increased resistance to apoptosis in cancer cells. Alterations in the ratio of bcl-2 to bax, a molecular rheostat controlling a cell's susceptibility to undergo apoptosis, seems to be one such mechanism. In addition, several recent studies showed that some transformed cells are not only resistant to FAS-induced apoptosis, but surprisingly, express FAS ligand, which was initially believed to be exclusively expressed by immunecompetent cells. This FAS ligand expression was documented in human colon cancer cells as well as hepatocellular cancers or melanomas (O'Connell et al. 1996, Strand et al. 1996, Hahne et al. 1996). The membrane bound FAS ligand on tumor cells was found to be functional and potently induced apoptosis in FAS-positive cells, such as lymphocytes. These observations led to the concept of the **FAS-counterattack**. Tumor cells thus evade elimination by the immune system through an active counterattack. In addition, these cancer cells are highly resistant to FAS, avoiding potential fratricide in an auto- or juxtacrine manner via this mechanism, as discussed above.

Other important examples of FAS ligand positive non-immune cells are found in sites of immune privilege, such as the anterior chamber of the eye or the testis (Nagata 1997, Bellgrau et al. 1995, Griffith et al. 1995). One characteristic of these extremely cautious tissues is that they do not tolerate normal immune reactions due to the risk of non-specific damage to nearby tissues. It was long believed that immune privilege is maintained by preventing activated immune-competent cells from entering these organs. Recently, evidence was put forward that although activated inflammatory cells can migrate into these organs, they immediately undergo apoptosis by engagement of FAS (Nagata 1997, Bellgrau et al. 1995, Griffith et al. 1995). In fact, constitutive expression of functional FAS ligand was reported in the corneal epithelium, iris and ciliary cells of the eye, as well as in Sertolli cells in the testis (Bellgrau et al. 1995, Griffith et al. 1995). These observations and the proposed FAS-counterattack of tumor cells led to the idea that FAS ligand expression on transplants might prevent allograft rejection. Lau et al. (1996) reported that myoblasts engineered to express FAS ligand significantly delayed

rejection of pancreatic β-cells when co-transplanted. These encouraging results, could not, however, be confirmed with Langerhans β-cells directly transfected with an adenoviral vector coding for the FAS ligand prior to transplantation into diabetic mice (Kang et al. 1997). In fact, rejection by a T or B cell dependent mechanism did not occur in these transplants, as expected. More importantly, however, these transplants were rapidly rejected due to a massive infiltration of the Langerhans islets by neutrophils, resulting in complete destruction of the transplant. These recent experimental findings open the door to the potential role of FAS ligand-based immunotherapies in the future.

Other mechanisms of intestinal epithelial cell apoptosis

Activation of the intracellular apoptosis-machinery also occurs by means other than in response to stimulation of specific receptors containing a death domain, i.e. the p55-TNF-R or FAS. A great variety of cytotoxic agents, such as inhibitors of protein synthesis, chemotherapeutic drugs or anti-metabolites are known to induce apoptosis under certain conditions (Ossina et al. 1997, Medina et al. 1996). Short chain fatty acids (SCFA) form one such group of particular importance to the field of intestinal epithelial cell biology. These SCFA are the endproducts of anaerobic bacterial fermentation of undigested fibers and other nutrients arriving in the colon (Velazques et al. 1996, Young and Gibson 1995). SCFA, mainly butyrate, serve as important energy substrates of intestinal epithelial cells, which rapidly oxidize SCFA (Young and Gibson 1995). Recently however, evidence was put forward that transformed intestinal epithelial cells cannot use these SCFA as fuel. Rather, the un-metabolized butyrate is highly toxic to these cells (Velaquez et al. 1996).

Using Caco-2 cells as a model of colon cancer, we showed that physiological concentrations of butyrate (0.1-100 mM) inhibited cell proliferation and furthermore, significantly induced apoptosis. Butyrate induced apoptosis occurred only after a lag-interval of approximately 14-16h. Even with high doses (100 mM or higher), butyrate failed to induce Caco-2 cell apoptosis earlier during this interval, in keeping with previous reports based on leukemic cell models (Medina et al. 1996). Furthermore, protein synthesis inhibitors, such as cycloheximide, completely blocked this apoptotic effect of butyrate. These experiments indicate the necessity of protein-neosynthesis to trigger apoptosis. Butyrate is known to inhibit the enzyme histone deacetylase, leading to conformational changes of these structural DNA-proteins. This allows transcription of otherwise "masked" genes (Boffa et al. 1978). Our data led us to speculate that one of these newly transcripted gene products is responsible for the activation of the caspase cascade. The most likely candidate in the Caco-2 cell model might be one member of the bcl-2 gene family. In fact, we were able to detect an increased expression of bak, a potent pro-apoptotic member of this family after stimulation with butyrate. On the other hand, anti-apoptotic bcl-2 levels remained unaltered in response to butyrate, thus

shifting the absolute ratio of these bcl-2 members significantly towards a pro-apoptotic profile. Bak correlates best with intestinal epithelial cell apoptosis, as recently demonstrated in normal colonic tissue, as well as in the HT-29 and IEC-18 cell lines (Moss et al. 1996). It is likely that this bcl-2 family member is the most relevant in enterocyte apoptosis. The implication of bak in butyrate-induced Caco-2 cell apoptosis was confirmed after treatment with the protein synthesis inhibitor cycloheximide, which inhibited butyrate induced upregulation of bak-expression as well as the induction of apoptosis. Therefore, we speculate that the mitochondrial pathway activated via bak is essential to the initiation of colon cancer cell apoptosis in response to butyrate. Hague et al. (1997) recently confirmed that bak is implicated in butyrate-induced apoptosis of some, but not all colon tumor cells. However, overexpression of bcl-2, which forms a heterodimer with bak, did not neutralize or block butyrate-induced apoptosis in this model. Bcl-2 overexpression rendered tumor cells only resistent to apoptosis after stimulation with butyrate, if a bak-independent pathway was implicated. Taken together, these observations clearly show, that depending on the tumor cell model different pathways are implicated in butyrate-induced apoptosis. The importance of the bcl-2 family in tumorigensis was recently underlined by the work of Rosen et al. (1998). The oncogene ras clearly suppresses apoptosis via downregulation of bak, when it is overexpressed in IEC-18 cells. A physiological increase in bak-expression is necessary for intestinal epithelial cells to undergo anoikis (programmed cell death, induced in intestinal epithelial cells by detachment from the matrix). If this homeostatic process is disturbed, these cells fail to undergo physiological apoptosis and they are likely to undergo tumorous transformation.

To further unravel the molecular mechanisms of SCFA-induced Caco-2 cell apoptosis, which is clearly independent of the above described classical apoptosis-receptors and adapter molecules, we selectively blocked various caspases, known to be implicated in TNF and/or FAS-induced apoptosis. We observed that specific activation of caspase-3 was necessary to trigger Caco-2 cell apoptosis in response to butyrate. Inhibition of this particular caspase with an irreversible peptide-inhibitor completely abolished the apoptotic response to butyrate. Furthermore, western blot analysis confirmed the proteolytic activation of caspase-3, the dominant effector caspase in

various other cell systems (Nagata 1997, Green 1998, Salvesen and Dixit 1997). As expected, classical substrates of caspase-3, such as poly-(ADP-ribosyl) polymerase were efficiently degraded, further confirming our findings that butyrate-induced apoptosis was mediated via the classical apoptosis pathway. This caspase-mediated degradation of the DNA-repair system, as well as the liberation of the DNA-fragmentation factor 40 due to the degradation of its inhibitor DFF 45 (unpublished observations), result in the typical nuclear morphological features of apoptotic cell death. DFF 40, a very potent DNase forms a heterodimer with DFF 45 in the cytoplasms which inhibits the translocation and nuclease activity of this factor (see I. *Molecular mechanisms of apoptosis*). Upon induction of apoptosis, cleavage of DFF 45 results in liberation and activation of DFF 40 and finally the occurence of characteristic DNA fragments (Boldin et al. 1996). Taken together, these findings indicate, that the preformed but inactive intracellular apoptosis-machinery can be activated in colonic epithelial cells by a great variety of factors such as butyrate, independent of classical death receptors, such as FAS or p55 TNF-R.

Role of cytokines in mediating interactions between enterocytes and immune cells

There is increasing evidence that enterocytes play an active role in the modulation and perpetuation of mucosal immune reactions in concert with immune-competent cells. Cytokines are the major mediators of this intercellular interaction. Enterocytes themselves are able to secrete many different cytokines and factors which act on immune-competent cells (Jung et al. 1995, McGee et al. 1993, 1995, Stadnyk 1994). For example, in response to bacterial invasion, intestinal epithelial cells respond by secreting IL-6 and IL-8, chemokines which attract other immune cells into a particular intestinal segment. Confirming these findings, infection of colonic epithelial cell monolayers with the invasive bacteria resulted in an upregulation of mRNA expression of IL-8, TNFα, GM-CSF and monocyte chemotactic protein 1 (Stadnyk 1994, Jung et al. 1995, Schuerer-Maly et al. 1994, Gross et al. 1995). These cytokines and chemokines are important factors in the generation of an effective immune response. IL-8 is a potent chemokine, attracting neutrophils and T lymphocytes (Strieter et al. 1989, Larsen et al. 1989), TNFα can act as a very strong activator of lymphocytes, whereas GM-CSF prolongs the survival of these cells (Danis et al. 1991, Lopez et al. 1992). These finding support the hypothesis that enterocytes can greatly augment inflammation via their secretion of cytokines. In addition, TNFα, IL-1β or IFNγ themselves stimulate the production and secretion of IL-8, thereby acting as a self-enhancing loop to amplify the mucosal inflammatory response (Schuerer-Maly et al. 1994, Gross et al. 1995). In HT-29 cells, protein kinase C (PKC) activation by PMA markedly stimulated IL-8 production. However the stimulatory effect of TNFα or IL-1β did not require PKC or A, indicating different signalling pathways (Gross et al. 1995). The stimulation of IL-8 by TNFα and IL-1β depended on protein tyrosine phosphorylation, as the PTK inhibitors genistein and herbimycin blocked this stimulatory effect (Gross et al. 1995). TGFβ, TNFα and IL-1β act in synergy to enhance IL-6 secretion by enterocytes in IEC-6 cells (McGee et al. 1995). IL-6 has overlapping functions with TNFα and IL-1, such as the induction of the acute phase protein response and modulating B and T-cell response (Akira et al. 1990, Molmenti et al. 1993). Furthermore, IL-6 preferentially enhances IgA secretion by mucosal B cells, suggesting

its potential role as a mucosal protecting cytokine (Beagley et al. 1989, Fujihasji et al. 1991). The gene expression and production of IL-6 can be directly stimulated by bacterial toxins, such as cholera toxin (McGee et al. 1993).

Another mechanism of lymphocyte-enterocyte interaction is illustrated by the modulation of MHC class II antigen expression on enterocytes. Mature intestinal epithelial cells normally express MHC class II molecules, allowing them to act as antigen presenting cells, in a manner similar to 'professional' antigen-presenting cells (APC), such as dendritic cells or macrophages (Mayer and Shlien 1987, Bland and Kambarrage 1991). In contrast, immature crypt cells are class II negative (Bland and Kambarage 1991 Bland and Whiting 1993). To induce a specific immune response, lymphocytes have to be activated by the presentation of a distinct epitope in combination with MHC class II and other co-stimulatory molecules, such as B7.1 or B7.2. The functional role of class II antigen expression by mature enterocytes is only partially elucidated, since these cells do not express costimulatory molecules of the B7 group (Sandersen et al. 1993). Recently, Yio and Mayer (1997) demonstrated that intestinal epithelial cells express a different type of surface protein, the glycoprotein gp180, which can act as costimulator. This molecule, in combination with the antigen-presenting complex, is recognized by the TCR:CD8 receptor complex on suppressor T cells, which are selectively activated. This is an important molecular mechanism potentially explaining the phenomenon of oral tolerance.

An increased and/or aberrant MHC class II expression on intestinal epithelial cells might result in the generation of pathological activation signals for T lymphocytes, potentially leading to increased self-antigen presentation, inducing autoimmunity. In fact, markedly enhanced HLA-DR expression on enterocytes was observed in various inflammatory bowel disorders. In patients with celiac disease, enhanced MHC class II and *de novo* expression on immature crypt cells, normally class II negative, were reported (Arnaud-Battandier et al. 1986). Other immune-mediated bowel-disorders such as Crohn's disease and ulcerative colitis are also characterized by an increased HLA-DR expression on enterocytes (Mayer et al. 1991). Furthermore, in the presence of inflammation, decreased intestinal gp180 expression is observed in addition to increased

enterocyte class II expression (Toy et al. 1997). The subsequently reduced potential to activate T-suppressor cells may thus increase the risk for an altered activation status of T-lymphocytes, potentiating the dysregulation of the intestinal immune system.

We hypothesized that the increased and aberrant enterocyte MHC class II antigen expression during inflammatory processes might be induced by cytokines. As already discussed above, under these conditions, the intestinal mucosal immune system is pathologically activated with recruitment of many additional inflammatory cells to the site of inflammation. IFNγ is well known to induce MHC class II antigens in several other models. Our data confirmed this hypothesis in the bowel. *De novo* class II molecule expression on immature intestinal epithelial cells was induced by the proinflammatory cytokine IFNγ. Furthermore, its effect was synergistically enhanced by TNFα, which alone did not alter class II antigen expression. These findings are in keeping with the report of *de novo* MHC class II induction in rat ileal crypt cells (IEC-17 cell line) after incubation with supernatants of PHA-stimulated intraepithelial lymphocytes, normally class II negative (Cerf-Bensussan et al. 1984). Similar results were obtained with the HT-29 cell line (Lundin et al. 1987).

The observation of an unusually long lag period of IFNγ-induced upregulation of MHC class II surface expression suggests that protein neosynthesis is required for this effect. In fact, previous studies confirmed that inhibition of protein synthesis completely blocked class II induction by IFNγ (Blanar et al. 1988, Amaldi et al. 1989, Celada et al. 1989). Despite considerable efforts in the past, no IFNγ response elements could be found in MHC class II genes (Benoist and Mathis 1990), indicating that IFNγ acts in an indirect way, via intermediary molecules. However, none of the classical IFNγ-inducible factors binds to class II promotor sequences. The missing element in the chain of events was recently discovered by Steimle et al. (1994). They showed that CIITA, a protein implicated in constitutive class II gene expression and not expressed in class II negative cells, is induced by IFNγ. In monocytes, fibroblasts and melanoma cells, CIITA gene expression is upregulated by IFNγ, subsequently activating MHC class II gene transcription (Steimle et al. 1994). This multistep reaction explains the long lag phase of MCH class II expression after IFNγ stimulation, as we observed in IEC-6 cells. It was

proposed that the synergistic effect of TNFα on IFNγ-induced class II upregulation is also due to an increased transcription rate (Panek et al. 1992).

Very recently, we showed that glucocorticosteroids inhibit cytokine-induced MHC class II expression in intestinal epithelial cells (Ruemmele et al. 1999), indicating that immunosuppressive agents might interfere with this process. Some reports in the literature indicated that corticosteroids diminish the pathologically increased MHC class II expression encountered in inflammatory tissues (Jevnikar et al. 1992). Three different mechanisms potentially explaining this phenomenon could be discussed: 1) steroid induced downregulation of pro-inflammatory cytokine production by immune competent cells that normally induce class II antigens; 2) steroid induction of anti-inflammatory cytokines that inhibit class II antigens, or 3) a direct effect of steroids on intestinal MHC class II gene expression and subsequently glycoprotein production and expression. Our observation of dexamethasone-induced inhibition of class II expression in intestinal epithelial cell monolayers in the absence of immune cells provides evidence that steroids are directly involved in enterocyte class II regulation.

Steroids may directly affect MHC class II gene transcription. There is some evidence that the glucocorticoid-glucocorticoid receptor complex (GGRC) interacts with transcription factor(s) essential for MHC class II gene transcription, thereby inhibiting the specific promotor region (Celada et al. 1993). In the latter study, Celada et al. (1993) demonstrated that GGRC inhibits the binding of a specific DNA binding protein to the X box in the MHC class II gene in mouse B-lymphocytes. Alternatively, it is possible that steroids may interfere with CIITA, a protein implicated in both constitutive and IFNγ-induced class II gene expression, as discussed above (Steimle et al. 1994). Another potential mechanism for the glucocorticoid effect on intestinal epithelial cell class II expression is via the regulation of mRNA levels for class II transcripts. In support of this hypothesis, steroids were reported to decrease the expression of macrophage class II mRNA levels and their transcription (Fertsch-Ruggio et al. 1988).

Taken together, these findings demonstrate that glucocorticosteroids are important regulators of cytokine-induced intestinal epithelial cell MHC class II

expression in intestinal epithelial cells. Our study raises the concept that the therapeutic benefit of corticosteroids in immune-mediated inflammatory bowel disorders may in part be explained by a direct effect on intestinal epithelial cells, rather than solely its immunosuppressive role. Reduction of the abnormally increased intestinal MHC class II expression may contribute to the downregulation of activated immune-competent cells in the actively inflamed intestinal mucosa. These findings provide a further example in support of the concept that cytokine-mediated epithelial cell - immune cell interactions in the intestinal mucosa are of critical importance in health and in disease.

Conclusions and Future Directions

In summary, our data provide clear evidence that cytokines, such as the pro-inflammatory factors TNFα and IFNγ, are potent modulators of intestinal epithelial cell turnover as well as important mediators of the intercellular cross-talk that occurs within the intestinal mucosa. To guarantee homeostatic enterocyte cell turnover, a delicate balance of growth stimulatory versus growth inhibitory factors and, at the same time, pro-apoptotic versus anti-apoptotic mediators must be maintained. In immune-mediated bowel disorders, this balance is disturbed resulting in altered intestinal epithelial cell turnover, leading to tissue injury and to major functional derangements. Our findings revealed that the increased enterocyte apoptosis rate observed in actively inflamed intestinal tissue may be mediated via a direct effect of TNFα. An increased understanding of the molecular mechanisms controlling enterocyte apoptosis will potentially help develop new immune-modulatory drugs to treat IBD. However, many specific questions still remain to be addresses, such as the molecular effects of pro-inflammatory cytokines on specific events in the progression of the cell cycle, the interaction of the discussed stress-activated pathways and classical apoptotic pathways or the role of specific inhibitors of apoptosis in the turnover of intestinal epithelial cells. To date, only symptomatic therapeutic strategies exist, given the fact that the cause of these immune-mediated bowel disorders remains known. An attractive possibility would be the use of potent anti-apoptotic drugs in treating these disorders. Theoretically, several different approaches could be chosen: Treatment with neutralizing or inhibitory antibodies directed against 1) cytokines 2) their receptors, or alternatively 3) the use of inhibitors to mediators critical to the propagation of the initial pro-apoptotic signal. The first option is already under clinical investigation. Initial trials with chimeric antibodies directed against TNFα were relatively successful in achieving remission in otherwise-treatment resistant CD patients, encouraging further research in this field. Another elegant way to suppress exaggerated intestinal epithelial cell apoptosis would be with specific inhibitors of intracellular mediators, such as caspase-inhibitors. Our data showed that ICE-specific caspase inhibitors were very potent in suppressing TNFα-induced enterocyte apoptosis However, we were very surprised to observe at the same time a switch towards necrotic cell death in response to TNFα. These findings indicate that anti-apoptotic drugs must be examined with caution, since they might change an

apoptotic response to a necrotic form of cell death, thereby increasing the tissue injury. Further in vivo studies are needed to clarify the potential use of anti-apoptotic drugs in treating IBD.

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