Colon Carcinoma Cells Use Different Mechanisms to Escape CD95-mediated Apoptosis

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ABSTRACT

CD95(APO-1/Fas) is a cell surface receptor that, when oligomerized by natural ligand, CD95L, or antibody, confers an apoptotic signal to apoptosis-sensitive cells. Whereas CD95 is expressed in every colonocyte of normal colon mucosa, CD95 is down-regulated or lost in the majority of colon carcinomas.

To investigate the sensitivity to CD95-mediated apoptosis of normal and neoplastic colonocytes, we applied cross-linking CD95(anti-APO-1) monoclonal antibody to freshly isolated colon crypts and colon carcinoma cell lines and monitored apoptosis by DNA fragmentation and morphology.

Normal colonocytes were constitutively sensitive to CD95-mediated apoptosis. All carcinoma lines were constitutively resistant but were sensitized upon pretreatment with IFN-γ. Transcription blocking, protein synthesis, and export in carcinoma cells indicated that even low surface levels of CD95 were sufficient to efficiently transmit the signal. Despite low CD95 surface levels of non-IFN-γ-treated cells, actinomycin D, cycloheximide, and brefeldin A each sensitized all cell lines, but at different rates and kinetics. In this context, it was observed that a greatly delayed apoptotic response of SW620 cells was associated with the absence of antibody-induced CD95 capping. Phorbol 12-myristate 13-aceate inhibited CD95-mediated apoptosis by counteracting the IFN-γ, actinomycin D-, and cycloheximide-mediated but not the brefeldin A-mediated sensitization. This phorbol 12-myristate 13-aceate-induced protection against apoptosis was completely abolished by staurosporine and by a selective protein kinase C inhibitor, Goe 6983.

We conclude that, during malignant transformation, colonocytes acquire different mechanisms to escape CD95-mediated apoptosis. These include abrogation of CD95, inhibition of CD95 capping, and activation of ant apoptotic programs, both governed by and independent of protein kinase C.

INTRODUCTION

CD95(APO-1/Fas) is a M, 48,000 cysteine-rich type I membrane glycoprotein and a member of the TNF receptor family (1,2). Upon oligomerization of CD95 by antibody (3) or trimerization of this receptor by its natural ligand, CD95L, it confers an apoptotic signal to apoptosis-sensitive cells. CD95-expressing cells undergo apoptosis in 2–4 h, provided that they are apoptosis sensitive. CD95L is a M, 40,000 type II membrane protein that belongs to the tumor necrosis factor family of cytokines (4,5). Like other members of this cytokine family, CD95L exists in a membrane-bound and a soluble form (6). CD95L is expressed on and released from activated T cells (6–9) by proteolytic cleavage (10).

Outside the immune system, CD95 is constitutively expressed by a broad panel of normal epithelial cell types and by a more restricted panel of mesenchymal cells (11). Irrespective of their state of maturation and differentiation (i.e., localization within the mucosal epithelial compartment), normal colonocytes regularly express CD95 at the basolateral surface membrane (12). Under physiological conditions, apoptotic epithelial death occurs mainly at the luminal surface of the colon mucosa (13). In normal colon, CD95L transcripts have been located in a small subpopulation of scattered mononuclear cells of the lamina propria but not in colonocytes (14,15). The paucity and random-like tissue distribution of these CD95L mRNA-positive mononuclear cells makes it very unlikely that CD95-triggered signals contribute to the physiological elimination of senescent colon epithelium. In ulcerative colitis, however, CD95L-expressing inflammatory cells are highly increased in number, and some of them are in close vicinity of crypt enterocytes undergoing apoptosis (16). These in situ data suggest that CD95/CD95L-induced programmed death of colonocytes might account for the emergence of the so-called aphthoid epithelial lesions. To further substantiate this concept, it must be formally proven that nonepithelial colonocytes are sensitive to CD95-triggered apoptosis.

In vitro, colon carcinoma cells can be killed by CD95 antibody, but at low rates (17). Thus, colon carcinoma cell lines constitutively show relative resistance to CD95-inducible death. Pretreatment with IFN-γ had an apoptosis-sensitizing effect (18). Malignant transformation of colon epithelium is often accompanied by abnormally low levels of CD95 protein. We found by immunohistology that CD95-negative subpopulations, occurring only rarely in colon adenomas, are common in colon carcinomas. In 39% of colon carcinomas, CD95 expression was diminished and, in 48%, it was undetectable, leaving a mere 13% of colon carcinomas that expressed CD95 at normal levels (12). Thus, down-modulation or abrogation of CD95 on tumor cells might be a selection advantage and constitute a mechanism of immune evasion to CD95-mediated killing by T cells (19).

We and others showed that IFN-γ enhances the level of CD95 protein exposed at the surface membrane. This also applies to many tumor cells, including colon carcinoma lines (11,12,17,18). Owen-Schaub et al. (17) showed that the level of CD95 expression is not predictive of CD95 antibody-mediated cell killing. Thus, although expression of CD95 molecules is prerotive in principle (1), the state of apoptotic responsiveness to CD95 cross-linking might be more complex than a simple function of the number of CD95 surface molecules (17).

After having developed a system to prevent the early spontaneous apoptosis of freshly extracted normal colon epithelium, which is due to loss of β1 integrin-mediated adhesion (20), we were in a position to study comparatively the responsiveness of normal and malignant colon cells to CD95 triggering in vitro.

MATERIALS AND METHODS

Antibodies and Reagents. CHX, ActD, PMA, SSP, collagenase I, and PI were purchased from Sigma Chemical Co. (St. Louis, MO). IFN-γ was a gift from Knock AG (Ludwigshafen, Germany). BFA was obtained from Boehringer Mannheim AG (Mannheim, Germany). The PKC inhibitor Goe 6983...
(21) was synthesized by Gödecke AG (Freiburg, Germany). The mAbs CD21(IgF) and CD30(Ki-1) that were used as isotype-matched controls were obtained from Dako (Copenhagen, Denmark). CD74(BU45) (IgG1 isotype) was a generous gift from I. M. MacLennan (Birmingham, United Kingdom). CD95(anti-APO-1) mAbs of IgG1 and IgG3 isotypes were raised and characterized in the laboratory of one of us (P. H. K.; Ref. 3).

Cell Culture Conditions. The human colon carcinoma cell lines COLO 205, SW480, SW620, and HT-29, purchased from American Type Culture Collection (Rockville, MD), were incubated in 25-cm² tissue culture flasks at 37°C in a humified 5% CO² atmosphere. Cells were maintained in 1:4 RPMI 1640 (Life Technologies, Inc., Grand Island, NY); Iscove's medium (Biochim, Berlin, Germany), supplemented with 10% FCS (PAA, Linz, Austria), 5 mM L-glutamine, 100 units/ml penicillin, and 100 /µg/ml streptomycin. Cells were washed once in medium with supplements. Apoptosis was induced by cross-linking surface CD95 with 500 ng/ml CD95(anti-APO-1) of the IgG3 isotype (3). The antibody (0.5 /µg/ml) was added to the culture medium of either pretreated or untreated cells. Whenever used, the modulating drugs were applied at final concentrations as follows: IFN-y, 100 units/ml; ActD, 0.5 /µg/ml; CHX, 5 /µg/ml; BFA, 1 /µg/ml; PMN, 5 ng/ml; SSP, 35 /µM; and Goe 6983, 1 /µM.

Flow Cytometric Analysis. Immunofluorescence staining was performed in polystyrene round-bottomed tubes (Falcon, San Jose, CA). All dilutions and washings were performed in 50 /µl of Hank's salt solution containing 0.5% sodium azide, 1.5% HEPES (Biochim), and 2% FCS, referred to as FACS medium. Approximately 10⁶ cells per sample, suspended in 50 /µl of FACS medium, were incubated on ice with an equal volume of the appropriate dilution of the mAb. After 45 min, cells were washed twice in 500 /µl of cold FACS medium, and 2 /µg of goat antimouse IgG F(ab')₂ FITC conjugate (Jackson Immunoresearch Laboratories, West Grove, PA) were added and incubated for 45 min on ice. Cells were washed again twice and resuspended in 300 /µl of medium for flow cytometry containing 1 /µg/ml PI (Sigma). From each sample, the green fluorescence of 10⁶ viable cells was analyzed. Normal apoptotic dead cells were excluded from analysis by selectively gating the PI fluorescence and on forward and side scatter parameters. Ten thousand cells per sample were examined for each determination. Flow cytometry was performed on a FACScan cytometer using the LYSIS II software (Becton Dickinson, Mountain View, CA). For analysis of surface CD95 expression, CD95(anti-APO-1) of IgG1 isotype was used to minimize nonspecific background staining.

Flow Cytometric Determination of DNA Fragmentation. To quantify cells with advanced DNA degradation, we used a procedure described by Nicotelli et al. (22). In short, approximately 10⁶ cells per sample were gently resuspended in 500 ml of hypotonic fluorochrome solution containing 0.1% Triton X-100 (Sigma), 0.1% sodium citrate, and 50 /µg/ml PI. The cell suspensions were placed at 4°C in the dark overnight before flow cytometric analysis. Ten thousand events were examined for each determination. Percentage specific death is defined as: [percentage DNA fragmentation in the presence of CD95(anti-APO-1) (IgG3 isotype) with or without supplements] − [percentage DNA fragmentation of the control with or without supplements].

Isolation and Treatment of Colonie Crypts. Proctocolectomy specimens were obtained from five patients with sporadic colorectal carcinoma within 15 min following surgery. Patients were from the control cohort of a prospective study on ulcerative colitis (16), which had been approved by the Ethics Committee of the Universitäts-Klinikum Heidelberg and for which informed consent was obtained from each individual. Colonic crypts were isolated from noninflamed mucosa samples at least 10 cm distant from the tumor, according to the method described by Whitehead et al. (23). Because isolated crypts rapidly undergo spontaneous apoptosis unless they are efficiently exposed to extracellular matrix components (20), crypts were embedded in a collagen gel as described by Hallowes et al. (24). Crypts were subsequently incubated with either the 500 ng/ml CD95(anti-APO-1) (IgG3 isotype) or an isotype-matched control antibody CD30(Ki-1) at 1 /µg/ml. For assessment of apoptotic rates by flow cytometry, gels were digested using collagenase I for 10 min at 37°C to obtain free crypts/cells.

"Capping" Assay and Immunocytochemistry. To demonstrate visually the fate of CD95 surface molecules after administration of cross-linking IgG3 and nonoligomerizing IgG1 CD95 mAbs, 0.5 × 10⁶ cells were treated with either mAb at a concentration of 0.5 /µg/ml. For comparison, CD74(BU45) was administered at an identical concentration. We previously reported that binding of CD74 mAb rapidly leads to internalization of CD74 surface molecules (25). Incubation of mAbs for 1 h was carried out on ice. Subsequently, unbound mAbs were removed by washing twice in Iscove's medium at 4°C. Next, cells were warmed up and kept at 37°C to restore physiological conditions. Thereafter, cells were harvested at different times, and cytospin preparations were made using a cytocentrifuge (Shandon, Pittsburgh, PA). Cytospins were fixed in acetone for 10 min at room temperature, air-dried, and subsequently stained by immunocytochemistry as follows. To localize bound murine mAb, a polyclonal biotinylated sheep antibody to mouse immunoglobulin that was reactive with all isotypes and a streptavidin-biotinylated horseradish peroxidase complex (Amersham, Buckinghamshire, United Kingdom) was applied. Cytospins were incubated with these second- and third-step reagents at a dilution of 1:100 for 10 min each. All dilutions and washings were carried out in PBS (pH 7.4). A substrate solution containing 0.4 mg/ml 3-amino-9-ethylcarbazole (Sigma) prepared in 0.1 M acetate buffer (pH 5.0) with 5% N,N-dimethylformamide (Sigma) and 0.01% H₂O₂ was applied for 10 min at room temperature. The peroxidase reaction caused an intense red precipitate. A faint hematoxylin nuclear counterstain was done to improve morphology. Stained cytospin preparations were rinsed in tap water and mounted with glycerin gelatin.

RESULTS

Malignant Transformation of Colon Epithelium Is Associated with Acquired Resistance to CD95-mediated Apoptosis. Freshly isolated colon crypts were embedded in a collagen gel to circumvent spontaneous apoptosis. Crypts were then incubated with cross-linking CD95 mAb. Three h after CD95 triggering, the specific death rate (see "Materials and Methods") was around 40% (Table 1). In contrast, colon carcinoma cell lines HT-29, COLO 205, and SW620 did not show any specific effect of the CD95 antibody; the SW480 cell line repeatedly featured a marginal sensitivity in the range of around 2% specific death after 3 h of exposure to the antibody (Table 1). The specific death rate steadily increased in a time-dependent fashion, indicating that the CD95 signaling was operative but greatly delayed (see Fig. 5). Thus, this unselected panel of colon carcinoma cell lines proved absolutely (or relatively, in the case of SW480) resistant toward CD95-induced cell death, although each line expresses CD95 (12).

Resistance Is Not Explained by the Constitutively Very Low Levels of CD95 Surface Expression. It has repeatedly been shown that IFN-y increases CD95 surface expression and sensitizes cells toward CD95-induced apoptosis (11, 12, 18). Accordingly, preincubation of colon carcinoma cell lines for 24 h with IFN-y (100 units/ml) leads to a substantial shift of sensitivity: after 3.5 h of anti-APO-1 treatment, all cell lines showed specific death at different rates in the range of 30% in HT-29 cells (data not shown). Comparable rates were obtained for COLO 205 (Fig. 1) and SW480 (see below) cells. However, at this time, specific death rates in SW620 cells were still exceedingly low (see below). To determine whether sensitivity is the consequence of increased CD95 surface expression, the experiment was repeated, but cells were additionally pretreated with BFA, a drug that specifically blocks protein export (26). As expected, BFA pretreatment completely abolished the IFN-y-induced specific death.

Table 1: Effect of CD95 triggering on normal colon epithelium and colon carcinoma cells

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<th>Effect of 3 h of anti-APO-1 treatment</th>
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Fig. 1. A, CD95(APO-1/Fas) expression on COLO 205 colon carcinoma cells measured by flow cytometry (---, isotype-matched control). The histograms (-----) represent APO-1 expression of cells in control medium (a), after 24-h pretreatment with 100 units/ml IFN-γ (b), or after 24-h 1 μg/ml BFA treatment (c). The CD95 level of BFA-treated cells matched that of constitutive CD95 expression. The 24-h IFN-γ (100 units/ml)-stimulated cells (b) expressed CD95 at a significantly higher level. B, flow cytometric analysis of nuclear DNA fragmentation; apoptotic nuclei are represented by hypodiploid DNA events, measured by PI staining of DNA (see "Materials and Methods"). The identical experimental settings depicted above were chosen and CD95(anti-APO-1) mAb (0.5 μg/ml; IgG3 isotype) was added for 3.5 h (see "Materials and Methods"). DNA fragmentation in controls without antibody, stimulated with IFN-γ (a) and treated with BFA (b), IFN-γ-pretreated cells exposed to the antibody (c), and BFA pretreated cells exposed to the antibody (d). [Specific death rates (see "Materials and Methods") are 31% for IFN-γ-pretreated and 68% for BFA-pretreated COLO 205 cells.]

increase of surface CD95 expression (data not shown). Unexpectedly, pretreatment of COLO 205 cells for 24 h with BFA alone (i.e., without IFN-γ) led to a specific CD95-triggered death rate of 75% and, thus, dramatically increased the sensitivity toward CD95 triggering despite low CD95 surface levels. Next, we exposed HT-29 cells for 24 h to BFA alone. In this case, the overall death rate was around 75%, corresponding to a specific death rate of about 60% (data not shown). Comparable results were obtained for COLO 205 (Fig. 1) and SW 480 cells. Thus, the number of constitutively surface exposed CD95 molecules was sufficient for efficient signaling.

Reversal of Primary Resistance to CD95-mediated Apoptosis by Inhibition of Gene Transcription, Protein Synthesis, and Export. The observed sensitization by blocking protein export with BFA is in line with effects obtained by inhibition of protein synthesis with CHX (17, 27, 28) and of gene transcription using ActD (18, 28). Both drugs rendered colon carcinoma cells sensitive to CD95-mediated apoptosis. These three different modes of sensitization collectively suggest the existence of a life-sustaining mechanism in which one or more protective proteins are operative. To compare the effects that these three modes of inhibiting generation and translocation of proteins have in our system, COLO 205 cells were pretreated for 12 h with ActD, CHX, and BFA and subsequently exposed to CD95(anti-APO-1). After this time of preincubation, the specific death rate was around 25% in the presence of ActD, about 55% in the presence of CHX, and around 40% in the presence of BFA (Fig. 2). The same applied to HT-29 (data not shown), SW480, and SW620 cells. However, these effects were extensively delayed in the SW620 cell line (see below). Thus, all drugs per se sensitized the cell lines, resulting in an apoptotic responsiveness to CD95 cross-linking, which was in the order of that induced by IFN-γ (Fig. 2).

Indications for Diverse Intrinsic Mechanisms to Escape CD95-mediated Apoptosis. We reasoned that, if permanent de novo synthesis of protective proteins is mandatory to maintain resistance toward CD95-induced apoptosis, the effect of blocking transport should be faster than that of blocking cytosolic protein synthesis, and this should still be faster than inhibition of transcription. We have

Fig. 2. Apoptosis induced by 3-h CD95 triggering. Prior to administration of cross-linking CD95 mAb (0.5 μg/ml), COLO 205 cells were pretreated for 12 h with ActD (1 μg/ml), CHX (5 μg/ml), BFA (1 μg/ml), or IFN-γ (100 units/ml). Columns, specific CD95 mAb induced DNA fragmentation rates; bars, SDs, determined on the basis of five independent analyses.

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shown that BFA effectively inhibits endoplasmic reticulum-Golgi transport as early as 3 min after addition to the culture (29). CHX and ActD were observed to start acting efficiently about 30 min after administration (30). Thus, pharmacokinetics are in the same range, i.e., rapid, and are, therefore, negligible. To determine the kinetics of sensitization toward CD95-mediated apoptosis, these three drugs were added to COLO 205 cells for different periods of time before CD95 treatment. Death rates were determined 3 h after antibody addition. The results are plotted in Fig. 3. The sensitizing effect of ActD had a delay of about 1 h and, during the next 6 h, reached a specific death rate on the order of 30%. Longer exposures proved toxic and nonspecific death rates increased significantly (data not shown). The first sensitizing CHX effect was seen after 30 min pretreatment prior to addition of antibody and rapidly increased during the first 3 h of preincubation, to attain a plateau at about 72% specific death after 4 h of pretreatment. The sensitizing effect of BFA was first observed after about 3 h pretreatment; after 7 h, the specific death was about 30%. At prolonged preincubation, the rates of specific death slowly increased and reached a level of about 70% after 24 h. These data indicate that blocking cytosolic protein synthesis acted faster than did inhibition of endoplasmic reticulum-Golgi transport. However, we ultimately attained comparable levels of sensitization. Seven h of pretreatment caused comparable levels of sensitivity in the case of ActD and BFA, which were significantly below that of CHX (Fig. 3). These results suggest diverse protective mechanisms impeding the CD95-effector cascade.

A Central Role for PKC in Resistance to CD95-mediated Apoptosis. In this study, several different authors have proposed a role for PKC in programmed cell death (27, 31) and, more specifically, in CD95-mediated apoptosis (28). Moreover, the apoptosis-regulating effect of PKC was shown to be dependent on de novo protein synthesis (32). To study whether protein synthesis and transport interfered with this hypothetical PKC effect, we first reevaluated the role of PKC in our system. To this end, 72-h IFN-γ-pretreated COLO 205 cells were exposed to PMA, the broad-spectrum (Ser) protein kinase inhibitor SSP, and Goe 6983, a compound with high PKC specificity, respectively (21), alone and in combination with PMA. The data are shown in Fig. 4B. The CD95-induced specific death rate of about 50% at 2.5 h was reduced to about 20% by 30 min of preincubation with PMA before antibody administration. This PMA effect was extensively antagonized by SSP and completely abolished by Goe 6983. Goe 6983 did not significantly modify the CD95-induced specific death rate per se (Fig. 4B) and, at least at the dosage applied and within the observation interval, neither inhibitor was toxic by itself (data not shown). Therefore, these observations clearly indicate that PKC is involved in the regulation of sensitivity to CD95 cross-linking. Next, we compared the effect of 1 h of PMA treatment on the sensitizing effects of IFN-γ, ActD, CHX, and BFA by taking the most effective time of preincubation for each sensitizing compound. The data are given in Fig. 4A. PMA extensively antagonized the IFN-γ-induced and partially antagonized the ActD- and CHX-induced sensitization but did not affect the sensitizing effect of BFA. In this experimental setting, each of these PMA effects was abolished by coincubation with SSP or Goe 6983 (Fig. 4B), indicating that those were, indeed, PKC effects, which, interestingly enough, did not require gene transcription or protein synthesis.

Delayed Apoptotic Response of SW620 Cells Is Associated with the Absence of Antibody-induced CD95 Capping. SW480 and SW620 are closely related colon carcinoma cell lines from the same patient derived from the primary tumor and a lymph node metastasis, respectively (33). In our hands, the lines still have very similar karyotypes. However, without and with IFN-γ, SW480 cells carry more CD95 molecules on the cell surface than do SW620 cells (Fig. 5A). Compared to the specific death rate of IFN-γ-pretreated SW480 cells, the apoptotic responsiveness of IFN-γ-pretreated SW620 cells to CD95 triggering was very poor within the first 6 h after mAb administration. After 24 h, however, kill rates were roughly within comparable ranges (Fig. 5B). Moreover, upon pretreatment with CHX or BFA and 3 h after exposure to CD95 antibody, both cell lines featured significant increases in specific death rates, which, however, were greater for SW480 cells (Fig. 5C). Thus, resistance of SW620 cells toward CD95-induced apoptosis was relative and seemed essentially to consist of a delayed reaction to CD95 antibody binding. In an attempt to characterize further this functional difference, we exposed IFN-γ-pretreated SW480 and SW620 cells to nonoligomerizing IgG1 and cross-linking (hence, apoptosis-inducing) IgG3 isotypes of CD95 (anti-APO-1) and immunocytochemically visualized the fate of these bound mAbs. Antibody incubation was done at 4°C. Excess mAb was washed away with cold medium. Cells were then warmed up to 37°C and, thereafter, harvested at different times, fixed, permeabilized by acetone, and immunostained for mouse immunoglobulin (see "Materials and Methods"). In both cell lines, the IgG1 isotype of anti-APO-1 mAb was evenly distributed on the cell surface and did not induce apoptosis (Fig. 6). This pattern was unchanged during the time of observation (i.e., 3 h). Thus, cells did not internalize CD95 antigen-CD95 antibody complexes. These observations were further substantiated by an isotype-matched internalization control: antibody-labeled CD74 molecules, known to be internalized (25, 29), were detected in cytoplasmic endosomal vesicles of both SW480 and SW620 cells, as early as 10 min after restoring 37°C culture conditions (Fig. 6). In SW480 cells, the IgG3 isotype anti-APO-1 mAb induced apoptotic morphology, which emerged 20 min after warming up. Apoptotic changes were preceded by a cap-like condensation of IgG3 antibody-labeled surface CD95 (Fig. 6). This capping pattern...
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Fig. 4. Influence of PKC stimulator PMA and PKC inhibitors SSP and Goe 6983 on CD95-triggered apoptosis in COLO 205 cells. COLO 205 cells were preincubated with IFN-γ (100 units/ml), ActD (0.5 μg/ml), CHX (5 μg/ml), or BFA (1 μg/ml), each for the maximum effective times, which were 72, 6, 4, and 20 h, respectively. PMA (5 ng/ml), with or without SSP (35 μM), or Goe 6983 (1 μM) was added 30 min (A) and 1 h (B), respectively, before exposure to cross-linking CD95 mAb (0.5 μg/ml). A. PMA pretreatment, which, per se, proved, to a minor extent, cytolytic in IFN-γ- and ActD-pretreated cells (death rates of PMA controls added to percentage nonspecific death I reduced specific, CD95-induced death rates in IFN-γ-, ActD-, and CHX-pretreated cells but failed to affect the BFA effect. B. The above PMA effects were largely reversed by SSP and completely abolished by Goe 6983. [Because SSP and Goe 6983 acted efficiently only after at least 1 h of pretreatment, PMA had to be administered for the same period of time. One h of PMA treatment was, per se, less effective than 30 min (data not shown). Therefore, the inhibition rate in B was lower than that in A.]

Discussion

We have shown that normal colon epithelium not only constitutively expresses CD95 but is also sensitive to CD95-mediated apoptosis. Considering our observation that CD95L transcripts are not expressed in normal colonocytes (14), it is very unlikely that normal colonocytes die by autocrine suicide [“cis-death,” according to Nagata and Golstein (34)], as was proposed for mature T lymphocytes after prolonged activation (34–36). The biological role of CD95 expression in normal colonocytes, therefore, very likely consists of maintaining susceptibility to T cell-mediated cytotoxicity. A “trans-death” of this kind may occur in the case of cognate interaction between an antigen-presenting colonocyte and an antigen-specific, CD95L-expressing cytotoxic T lymphocyte. Through this, as yet, hypothetical mechanism, targeted, e.g., virally infected, colon epithelia might be eliminated. Colonocyte apoptosis may alternatively occur via ligation of soluble CD95L (7), released by activated, CD95L-expressing mononuclear cells of the lamina propria (14, 15). This type of enforced suicide of “innocent bystander” colonocyte has been proposed for the early epithelial lesion in ulcerative colitis (16). There is in situ evidence that colon adenomas express CD95 at high levels (12) and also feature high apoptotic rates within the neoplastic component (13). Whether the CD95/CD95L system is involved in programmed death of adenoma cells is, as yet, unclear, as is whether CD95-mediated death occurs in colon carcinoma cells in vivo.

Immunohistology revealed that the majority of colon carcinomas express CD95 at abnormally low levels or entirely lack CD95 (12). Further, our data confirm that colon carcinoma cell lines are constitutively or, at least, relatively resistant to CD95-mediated apoptosis (17, 18). These findings imply that, during malignant transformation, colon carcinoma cells down-modulate or abrogate CD95 and/or acquire (relative) resistance to CD95 ligation.

It seems likely that structural and/or functional defects due to genomic deterioration contribute to these processes. Paradigmatically, the lack of cap formation upon CD95 ligation by cross-linking CD95 mAb that we observed in SW620 cells might be the consequence of disturbed lateral membrane mobility or cytoskeletal assembly or of a lack of capping proteins. In either case, in the absence of cap formation on SW620 cells, cross-linking CD95 mAb, alone and in conjunction with sensitizing agents, acted with a delay on the order of several hours, as compared to SW480 cells, which capped readily.

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We and others (17, 18, 37) have shown that, in vitro, resistance to CD95-mediated apoptosis can be overcome by administration of IFN-γ. This activation-induced sensitization appeared not to depend on only the well-known enhancement of CD95 surface expression, which we show is not mandatory for effective signaling. Its critical 6-h delay in action (Fig. 3) suggests that IFN-γ induces a protein or proteins that act as an agonist or agonists in the effector cascade. Three such IFN-γ-induced, programmed cell death-promoting proteins have recently been found in HeLa cells: a novel Mr 15,000 protein, DAP-1; a novel Mr 160,000 calmodulin-dependent serine/threonine kinase, DAP kinase (38); and cathepsin D protease (39).

Malignant transformation-associated resistance to CD95-mediated apoptosis might be achieved by activation of abnormal or surplus cellular programs orchestrating life sustaining mechanisms. The concept of protective proteins was promoted by Ni et al. (28), who observed an apoptosis-sensitizing effect of ActD and CHX pretreatment on anti-Fas antibody-mediated killing in mouse hepatocytes. Confirming and extending these data in human colon carcinoma cells, we have shown that even low amounts of CD95 surface molecules are sufficient to mediate the apoptotic signal, provided that gene transcription or cytosolic protein synthesis or transport from endoplasmic reticulum to the Golgi is specifically inhibited. Our data point to the existence of a complex antiapoptotic machinery that is crucially dependent on a steady de novo protein supply. The rapid effects of CHX and BFA (see Fig. 3) suggest a very short half-life for such putative antiapoptotic proteins.

Theoretically, the CHX effect might also be explained in a different, perhaps complementary, manner. CHX has been shown to elevate and stabilize mRNA levels of c-Myc (30). Under conditions that are not yet well understood, elevated MYC levels rapidly induce apoptosis (40, 41). It will have to be shown whether CHX treatment causes MYC overexpression in our system.

Work on the cytolytic signal cascade following binding of TNF to its receptor yielded data pointing to the existence of protective proteins that confer TNF resistance. Among them are ROI-scavenging enzymes like manganese superoxide dismutase and mitochondrial O2−-scavenging enzyme [reviewed by Jäättelä et al. (42)]. Based on classical studies [reviewed by Clément and Stamencovic (43)] showing that a peroxidant state is a common feature in tumor cells, it was attractive to address the role of ROIs in resistance toward CD95-mediated apoptosis. Although Hug et al. (44), working in a human CD95-transfected murine fibroblast cell line, did not detect any modulating effects of antioxidants on CD95-mediated apoptosis, ROIs might, nevertheless, be important modulators of CD95-mediated apoptosis.
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Fig. 6. Immunomorphology of IFN-γ-pretreated SW480 (left) and SW620 (right) colon carcinoma cells exposed 30 min at 4°C to noncomplexing (IgG1 isotype) and complexing (IgG3 isotype) CD95(anti-APO-1) mAb and to a rapidly endocytosed control mAb CD74(BU-45) (IgG1 isotype). Cells were warmed to 37°C and were harvested after the indicated elapsed times, acetone-fixed, and immunostained for bound murine Ig. Morphological details are described in "Results." [Aminoethylcarbazole immunolabeling (red) and hematoxylin nuclear counterstain; bar, 30 μm.]

apoptosis-sensitivity in malignant cells. Clément and Stamencovic (43) have shown that high intracellular O$_2^-$ concentrations can block CD95-triggered apoptosis.

We showed that enhanced PKC activity partly abolishes the sensitizing effect of IFN-γ and up-regulates protective mechanism(s) that are inhibited or antagonized by ActD and CHX (Fig. 4). PKC represents a family of at least 10 phospholipid-dependent isoenzymes that catalyze the phosphorylation of proteins at serine and threonine and act in various steps of intracellular signal transduction. Diacylglycerols and phorbol esters activate PKC. The most potent PKC inhibitor
known thus far is SSP. However, this inhibitor lacks specificity for PKC, in that it also suppresses cAMP-dependent kinase and tyrosine kinases [reviewed by Gschwendt et al. (21)]. The SSP derivative Goe 6983 is more specifically directed at PKC (21). Using both inhibitors, we show that the PMA effects are, in fact, due to enhancement of PKC activity. In our system, PMA acts relatively fast, i.e., in less than 30 min (Fig. 4). PKC activity negatively interferes with a ceramide-initiated Ras signaling pathway operating in CD95-triggered death (45). This Ras pathway is initiated by CD95 ligation-induced activation of sphingomyelinase, leading within 30 s to elevated cytosolic ceramide levels (46, 47), followed by a ceramide-mediated Ras activation which was detectable after 2 min (45). Against the background of the known complexity of PKC action, dissection of the Ras pathway is unlikely to be its only effect. Clément and Stamnenovic (43) studied the superoxide-producing enzyme activity in TNF-CD95 transfected M14 melanoma cells. Upon PMA treatment, these authors found elevated O$_2^-$ concentrations in parallel to decreased CD95-mediated apoptosis. This suggests a short-lived, cytosolic, protective protein that is not interfered with the apoptosis-sensitizing effect BFA had in our system. Interestingly, however, PKC did not interfere with the apoptosis-sensitizing effect BFA had in our system. This suggests a general mechanism for regulating organelle structure and membrane traffic. Cell, 67: 601–616, 1996.


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Colon Carcinoma Cells Use Different Mechanisms to Escape CD95-mediated Apoptosis


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