Increased p53-dependent Apoptosis by the Insulin-like Growth Factor Binding Protein IGFBP-3 in Human Colonic Adenoma-derived Cells

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Abstract
We investigated the expression of insulin-like growth factor binding protein 3 (IGFBP-3) in normal human colonic epithelium and whether IGFBP-3 is involved in the induction of apoptosis in colonic epithelial cells. A gradient of IGFBP-3 protein expression was observed within the normal colonic crypt, and increased IGFBP-3 expression was coincident with the region of increased differentiation and apoptosis. Treatment of human colonic tumor cell lines with IGFBP-3 alone was shown to have no effect on growth. However, an increase in p53-dependent apoptosis was observed in the presence of 100 ng/ml IGFBP-3 24 h after the induction of DNA damage by γ-irradiation. These results suggest that IGFBP-3 enhances the p53-dependent apoptotic response of colorectal cells to DNA damage.

Introduction
In a constantly self-renewing tissue such as the colon, the regulation of cell numbers must be strictly controlled. Tissue homeostasis in the normal colonic crypt relies on a balance between proliferation, differentiation, and apoptosis, with apoptosis occurring at the top of the colonic crypt as the culmination of a differentiation pathway (1, 2). The aim of this investigation was to determine whether IGFBP-3 is involved in the regulation of apoptosis in human colonic epithelial cells. Although IGFBP-3 is known to inhibit cell growth by sequestering IGF, there is growing evidence that IGFBP-3 can have a direct effect on cell growth that is independent of IGF-1. In support of its role as a primary growth inhibitor, IGFBP-3 can be increased by cell growth inhibitory agents such as TGF-β (3). Using a p53 and IGF-1 receptor-negative prostatic cell line, Rajah et al. (3) demonstrated that the addition of IGFBP-3 resulted in increased apoptosis. In addition, treatment with TGF-β (known to induce apoptosis in this PC-3 prostatic cell line) caused an increase in IGFBP-3 6–12 h before the onset of apoptosis, which led to the conclusion that IGFBP-3 induces apoptosis through a novel pathway independent of p53 or IGF-1. In addition, TGF-β has also been reported to increase IGFBP-3 secretion in neonatal human fibroblasts (4) and human breast cancer cells (5). Furthermore, senescent fibroblasts have been shown to secrete abnormally high concentrations of IGFBP-3, suggesting a link between IGFBP-3 accumulation and loss of proliferative capacity (6). Growth inhibition of human breast cancer cells MCF-7 (IGF-1 responsive) by retinoic acid treatment resulted in the appearance of IGFBP-3 in cell conditioned medium (7). IGFBP-3 is also reported to inhibit the growth of the IGF-1 nonresponsive breast cancer cell line HS678T (8). In addition, IGFBP-3 has been shown recently to increase ceramide-induced apoptosis in this cell line, although there was no induction of apoptosis by the addition of IGFBP-3 alone (9). Therefore, because IGFBP-3 has been implicated to have a role as a proapoptotic protein, the aim of this study was to determine whether IGFBP-3 is involved in the induction of apoptosis in colonic epithelial cells. El Atiq et al. (10) reported increased IGFBP-3 in the serum from colorectal cancer patients (however, circulating IGFBPs may originate from the liver). In contrast, Michell et al. (11) failed to detect any secreted IGFBP-3 in primary cultures of normal and malignant colonic epithelium, despite consistent detection of mRNA for IGFBP-3 and the identification of immunoreactive IGFBP-3 in purified colonocytes in culture. Therefore, initially we wished to establish whether IGFBP-3 was expressed within the normal colonic crypt and if so, whether a gradient of expression was maintained, as shown for important growth factors such as TGF-β (12). In addition, we wished to determine whether expression of IGFBP-3 was deregulated in colonic tumor tissue. The aim was then to determine the functional consequence of IGFBP-3 secretion for colonic epithelial cell survival, whether IGFBP-3 is involved in the induction of apoptosis in colonic epithelial cells, and whether loss of response to IGFBP-3 occurs in colorectal carcinogenesis.

Materials and Methods

Immunostaining of Normal Human Colonic Crypts and Colonic Tumor Tissue. Sections were prepared from archival material that comprised formalin-fixed, paraffin-embedded tissues retrieved from the files of the Department of Histopathology, Bristol Royal Infirmary. Eleven colonic adenomas of various sizes and 11 colo rectal adenocarcinomas (ranging from well differentiated to poorly differentiated) were examined. Normal mucosa was obtained from resection margins at least 6 cm from the tumor mass (5 specimens) and tissue adjacent to the tumors where appropriate (of normal appearance in H&E sections). Staining was performed on 5-μm formalin-fixed, paraffin-embedded tissue using a standard avidin-biotin peroxidase technique and pretreatment with high temperature (pressure cooker) antigen retrieval using 10 mM citrate buffer (pH 6.0). The in-house IGFBP-3 antibody was applied at a dilution of 1:800. Slides were assessed by two independent observers.

Cell Lines and Culture Conditions. ANC1 and BH/C1 are clonogenic, nonmutogenic human adenoma-derived cell lines that express wild-type p53 and were grown in conditioned medium. RGC2 is a clonogenic, nonmutogenic human adenoma-derived cell line hemizygous for the p53 gene, with the remaining allele having a 262 (Arg→Trp) mutation and grown in 20% FBS DMEM. PC/JW is a human carcinoma derived cell line that is null for p53 protein expression, routinely cultured in 10% FBS DMEM.

Treatment with 5 Gy of γ-Irradiation ± IGFBP-3. Cells were seeded at 2 × 10^5 per flask in duplicate flasks and grown under standard growth conditions until ~60% confluent. Preliminary investigations showed that all of the cell lines investigated were able to tolerate serum-free growth conditions (SFM) well for up to 96 h (SFM is standard, nonconditioned growth medium conditioned with 10% FCS).
without the addition of FBS). Therefore, cell lines were grown for 24 h in SFM to remove IGFBP-3 present from the serum and then grown for 24 h in SFM supplemented with or without 100 ng/ml IGFBP-3 (effective reported dose; Ref. 9). The cells were then exposed to 5 Gy of γ rays from a 137Cs source at a dose rate of 0.33 Gy/min in SFM supplemented with 20 mM HEPES (Sigma) for buffering (±IGFBP-3), and attached and floating (apoptotic, refer below) cell yields were determined up to 48 h after irradiation.

Assessment of Apoptosis. The level of apoptosis in cultured epithelial cell lines was assessed by measuring the proportion of cells that detached from the monolayer and were floating in the medium and by determining the fraction of these floating cells that were apoptotic. The attached and floating cell populations were stained with 5 µg/ml acridine orange in PBS and analyzed by fluorescent microscopy for morphological features of apoptosis [most obviously the characteristically condensed chromatin, an example of which is shown in Fig. 8 and has been described previously by Gregory et al. (13) and Hague et al. (14)]. Analysis was carried out by an experienced observer unaware of the cell type or treatment. The fraction of floating cells that were apoptotic did not significantly vary between treated and control untreated cell populations (between 85 and 95% for RG/C2 cells, for example), and therefore the number of floating cells could be used as a measure of the induction of apoptosis.

Treatment with 5 Gy of γ-Irradiation ± αIR3 Antibody. To verify that any effect of IGFBP-3 was not attributable to sequestration of IGF-1, cells were incubated in SFM for 24 h, followed by SFM plus the anti-IGF-1R antibody αIR3 (Oncogene Research Products) at concentrations of 1 and 5 µg/ml (15) for 24 h and then irradiated (5 Gy). The cell yield and floating cell population in the presence of the αIR3 antibody were determined 24 and 48 h after irradiation.

Results and Discussion

When studying the expression of IGFBP-3 in the normal colonic epithelium, differential staining was observed within the crypt. IGFBP-3 staining was found to be cytoplasmic; the cells in the top half of the crypt and at the lumenal surface were more intensely stained than those at the bottom of the crypt. Increased IGFBP-3 expression was, therefore, found to be coincident with the region of increased differentiation and apoptosis within the normal colonic crypt (Fig. 1A). In addition, in both adenoma and carcinoma tissue, this differential expression was disrupted, with all epithelial-derived tissue showing positive staining for IGFBP-3 (Fig. 1B). Interestingly, this pattern of expression was similar to that observed for TGF-β, with the gradient of expression being disrupted in tumor tissue (12). In the three poorly differentiated carcinomas (of the 11 adenocarcinomas studied), there was noticeably less staining for IGFBP-3, again suggesting that there may be an association between IGFBP-3 expression and differentiation (Fig. 1C). Therefore, IGFBP-3 expression was found to be differentially regulated within the normal colonic crypt (its location was associated with the region of differentiation and apoptosis; Refs. 1 and 2), and this regulation appeared to be disrupted in tumorigenesis. Because this was similar to the pattern of staining observed previously for TGF-β, a potent growth inhibitor for colonic epithelium (16, 17), this suggests that IGFBP-3 may have an important role in the regulation of differentiation and apoptosis in human colonic epithelium.

Therefore, because IGFBP-3 has been shown to be expressed in the colon in vivo and is growth inhibitory in a number of different cell systems, the effect of IGFBP-3 on colonic cell growth in vitro was investigated. The cell lines investigated were three clonogenic, non-tumorigenic human adenoma-derived cell lines, AN/C1, BH/C1, and RG/C2, and one carcinoma-derived tumorigenic cell line, PC/JW2. The adenoma-derived AN/C1 and BH/C1 cell lines express wild-type p53, whereas the RG/C2 cell line is hemizygous for the p53 gene; the remaining allele has a 282 (Arg→Trp) mutation. The carcinoma-derived PC/JW cell line is null for p53 protein expression. Cell lines were grown for 24 h in SFM to remove IGFBP-3 present from the

Fig. 1. Tissue sections stained with IGFBP-3 antibody. A, normal colonic epithelium showing differential staining for IGFBP-3; the more intense staining is located toward the top of the colonic crypts and at the luminal surface. B, adenoma tissue showing intense staining for IGFBP-3 throughout the epithelial tissue, indicating loss of the expression gradient present in the normal colonic crypts. C, an undifferentiated adenocarcinoma showing weak staining for the IGFBP-3 protein but including a more differentiated region showing more intense staining.
serum and then grown for up to 3 days in SFM supplemented with 100 ng/ml IGFBP-3 [as reported by Gill et al. (9)]. Preliminary investigations had shown that all of the cell lines tolerated serum-free growth conditions well for up to 96 h. For all cell lines tested, the addition of IGFBP-3 to the culture medium had no effect on cell growth (either cell yield or number of detached floating cells, which were confirmed to be apoptotic; refer below) as compared with the control cultures grown in SFM alone (results not shown). Therefore, the addition of IGFBP-3 to the tissue culture medium had no effect on cell growth or rate of apoptosis in any of the adenoma- and carcinoma-derived cell lines tested, regardless of p53 status. This finding was similar to that reported by Gill et al. (9), where neither significant growth arrest nor induction of apoptosis by IGFBP-3 was seen in the Hs578T breast cancer cell line. In contrast, the addition of IGFBP-3 was sufficient to induce apoptosis in the GBM cell line (18) and in the PC-3 prostate cancer cell line (3), although both used 500 ng/ml recombinant IGFBP-3. Therefore, the lack of response of the colonic epithelial-derived cell lines may be attributable to the lower concentration of recombinant IGFBP-3 used in this study (100 ng/ml), although it should be pointed out that Gill et al. (9) showed no response at 500 ng/ml IGFBP-3 in the Hs578T breast carcinoma cell line (9). It is possible that the variation in cellular response to IGFBP-3 may reflect tissue-specific differences in the regulation of apoptosis.

However, because IGFBP-3 has been shown to be transcriptionally regulated by p53 in EB1-transfected colon carcinoma cells (19) and to enhance ceramide-induced apoptosis in a breast carcinoma cell line (9), we wished to address the question as to whether secreted IGFBP-3 could affect the p53-dependent apoptotic response of colonic epithelium-derived cell lines to cellular stress such as that resulting from DNA damage. Therefore, for these experiments, 100 ng/ml recombi-

Fig. 2. Cell survival of cells expressing wild-type p53 function up to 48 h after exposure to 5 Gy of γ-irradiation as shown by: attached cell yield (A, BH/C1 cell line, passage 99; C, AN/C1 cell line, passage 107); and induction of apoptosis, as shown by floating cells expressed as a percentage of the total cell population (where the floating cells are confirmed to be apoptotic; see “Materials and Methods”; B, BH/C1 cell line, passage 99; D, AN/C1 cell line, passage 107). The addition of 100 ng/ml IGFBP-3 to nonirradiated cell cultures had no significant effect on cell survival as measured by attached cell yield or the floating cell population (results not shown). The results shown represent the means of duplicate experiments; bars, SD.
nant IGFBP-3 was added to two adenoma-derived cell lines (BH/C1 and AN/C1), which secrete relatively low levels of the IGFBP-3 protein (data not shown) and express wild-type p53 protein. Previously, although induction of a p53-dependent G1 arrest could be detected in cell lines with wild-type p53 expression after exposure to 5 Gy of γ-irradiation (20), significant rapid p53-dependent apoptosis had not been detected in any of these cell lines. In addition, the cellular response of the adenoma-derived cell line RG/C2 (282 Arg→Trp p53 mutation) and the carcinoma-derived PC/JW2 cell line (which is null for p53 protein expression) was also investigated. All cell lines were grown in SFM for 24 h, followed by SFM + 100 ng/ml IGFBP-3 for 24 h, and were then irradiated with 5 Gy. The cultures were maintained in SFM ± IGFBP-3 for up to 48 h (prior to the onset of p53-independent apoptosis), and attached and floating cell yields were determined. As we and others have described previously, the level of apoptosis in cultured epithelial cell lines can be assessed by measuring the proportion of cells that have detached from the monolayer and are floating in the medium and by determining the fraction of these floating cells that are apoptotic using acridine orange staining to confirm morphological features of apoptosis (14, 21).

The results show that in the presence of exogenous IGFBP-3, there is a >30% decrease in cell yield and increase in apoptosis in the wild-type p53-expressing cell lines (BH/C1 and AN/C1) after exposure to 5 Gy of γ-irradiation (Fig. 2). To verify that this induction of apoptosis was not attributable to sequestration of IGF-1 (a potent survival factor) by the addition of IGFBP-3, the anti-IGF-1R antibody, αIR3, was added to irradiated cultures, and the cell yield and floating cell population was determined at 24 and 48 h after irradiation (Fig. 3). In both the AN/C1 and BH/C1 cell lines, addition of the αIR3 antibody had no effect on cell yield or apoptosis of the irradiated.
cultures. Therefore, the induction of apoptosis by DNA damage in the presence of IGFBP-3 was independent of its effect on the IGF-1 pathway. In addition, this induction of apoptosis appears to be dependent on the expression of wild-type p53 because no change in cell yield or induction of apoptosis was observed in either the mutant p53-expressing RG/C2 or p53-null JW/2 cell lines (Fig. 4).

Therefore, in the presence of DNA damage, the addition of recombinant IGFBP-3 was shown to cause an ~2-fold increase in p53-dependent apoptosis in the colorectal epithelium-derived cell lines after 48 h. This induction of cell death after DNA damage was similar to that reported for the Hs578T breast carcinoma-derived cell line after treatment with a ceramide analogue (9), although the Hs578T cells have been shown to be mutant for p53 expression, suggesting that the potentiation of cell death was independent of p53 function in this breast carcinoma cell line. In contrast, in the current investigation, induction of apoptosis was limited to those cell lines that express wild-type p53 protein. The lack of apoptotic response in the p53-null cell line showed that this was not related to expression of a mutant p53 protein. Therefore, our findings suggest that expression of wild-type p53 protein is necessary for IGFBP-3-induced apoptosis after irradiation of colonic epithelial cells.

Finally, the question arises as to the significance of these findings for colonic tumor cell survival. Previous studies suggest that as IGFBP-3 appears to act as a proapoptotic protein in a number of cell systems, loss of p53 function would lead to a down-regulation of secreted IGFBP-3 protein, potentially resulting in loss of apoptotic signals, and ultimately leading to aberrant cell survival. However, in addition, a recent report by Zou et al. (22) suggests that the DNA

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Z. P. Gill, personal communication.
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sequence of IGFBP-3 may be altered in both the normal and tumor tissue of individuals with gastrointestinal tumors and suggest that such changes may lead to a predisposition for developing gastrointestinal malignancy (22). Furthermore, Manousos et al. (23) suggest that the level of circulating IGFBP-3 may be inversely associated with risk for colorectal cancer. Our results suggest a potential mechanism by which such a predisposition may occur: Secretion of IGFBP-3 leads to growth inhibition (either through an autocrine or paracrine mechanism) via potentiation of p53-dependent apoptosis. Loss of IGFBP-3 function would therefore lead to loss of sensitivity to growth inhibition under conditions of cellular stress or DNA damage, resulting in the clonal expansion of cell populations that fail to express functional IGFBP-3 protein. The level of IGFBP-3 secretion may therefore contribute to the survival of adenoma cells, because loss of the IGFBP-3 response prior to acquisition of a p53 mutation could lead to clonal selection of the aberrant cell populations.

In summary, differential expression in the normal colonic crypt suggests an important role for IGFBP-3 in the regulation of colonic cell survival. In addition, IGFBP-3 has been shown to enhance p53-dependent apoptosis after DNA damage. Therefore, loss of IGFBP-3 could contribute to the development of colonic adenomas that retain wild-type p53 function through suppression of p53-dependent apoptotic signals, allowing aberrant cell survival and tumor formation. These results therefore suggest a possible role for IGFBP-3 in cancer prevention by sensitizing cells to p53-dependent damage/stress-induced apoptosis and hence inhibiting the development of adenomas while having no effect on surrounding, undamaged cells.

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References

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