THE aim of this study was to assess whether inter-leukin-10 (IL-10) and/or transforming growth factor β-1 (TGFβ₁) downregulate HLA-DR expression using the HT29 cell line as a model of colonic epithelial cells. HLA-DR expression was induced in HT29 cells with γ-interferon. The effects of IL-10 alone, TGFβ₁ alone, and IL-10 and TGFβ₁ in combination were studied. HLA-DR expression was assessed using flow cytometric analysis. γ-Interferon induced HLA-DR expression in a dose-dependent fashion. In the absence of γ-interferon, neither IL-10 nor TGFβ₁ induced HLA-DR expression. In isolation, neither IL-10 nor TGFβ₁ downregulated HLA-DR expression. When IL-10 and TGFβ₁ were added in combination, small (6–30%) statistically significant reductions in HLA-DR expression were seen. The biological significance is unclear.

Key words: Interleukin-10, Transforming Growth Factor β 1, γ-Interferon, HLA-DR, HT29 cell culture

The effect of interleukin-10 and transforming growth factor β-1 on HLA-DR expression in colonic epithelial cells

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Introduction

HLA-DR molecules are cell surface heterodimers that act as immune recognition molecules. A variety of antigen-presenting-cells present antigen in association with HLA-DR to lymphocytes. This interaction leads to lymphocyte activation and proliferation, and thus promotes inflammatory responses.[1] In addition to constitutive expression on a variety of cell types including lymphocytes, macrophages, vascular endo-thelium and some epithelial cells,[2] expression of HLA-DR molecules is induced in a much wider range of cell types in the presence of inflammation.[3]

Under normal conditions, HLA-DR molecules are not expressed by colonic epithelium,[4] however, colonic epithelial cell expression of HLA-DR is seen in a variety of inflammatory bowel diseases.[5,6] In vitro studies have shown that HLA-DR-bearing colonic epithelial cells can present antigen to mucosal lymphocytes.[7,8] It is thus likely that HLA-DR expression by colonic epithelial cells is an important step in the generation of mucosal immune responses.

The mechanisms that regulate colonic epithelial cell expression of HLA-DR are being defined. Induction of HLA-DR is mediated by pro-inflammatory cytokines such as γ-interferon.[3,9] γ-Interferon has been widely used to induce HLA-DR expression in human gastrointestinal carcinoma cell lines.[10–12] The

mechanisms by which colonic epithelial cell HLA-DR expression is downregulated when mucosal inflammation resolves have not been identified.

Some cytokines have immunosuppressive properties, some of which may be due to downregulation of HLA-DR expression in antigen presenting cells. Inter-leukin-10 (IL-10) and transforming growth factor β (TGFβ) are of particular interest. IL-10 has a variety of inhibitory actions including inhibition of T-lymphocyte activation/function as well as inhibition of the production of chemokines and cytokines.[13–15] IL-10 inhibits γ-interferon-induced expression of MHC Class II antigens in a variety of cell types.[16–19] Mice that are deficient in the IL-10 gene develop chronic enterocolitis.[20] TGFβ is present throughout the gastrointestinal mucosa[21] and has a variety of inhibitory functions such as inhibition of epithelial cell proliferation.[22] TGFβ also down regulates HLA-DR expression in a variety of cell types, including a colonic carcinoma cell line.[23] As with the IL-10 'knockout' mouse, mice with disrupted TGFβ genes develop multifocal chronic inflammation which includes involvement of the gastrointestinal tract.[24] With these observations in mind, we hypothesized that IL-10 and/or TGFβ downregulate colonic epithelial HLA-DR expression.

The aim of this series of experiments is to test whether IL-10 and TGFβ down-regulate γ-interferon-

induced HLA-DR expression in an experimental model of colonic epithelium. The cell line selected for use in this study (HT29/19a clone) is a well differentiated human colonic carcinoma cell line that forms monolayers with ultrastructural and functional similarities to normal colonic epithelium.[25–28] The HT29 cell line has been used in previous studies examining the effects of different agents on colonic epithelial cell expression of HLA-DR.[9,10,12]

Methods

Cell line

The HT29/19a colonic carcinoma cell line was a gift of Professor Laboisse.[26] The stock cultures were maintained at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with glucose 4500 mg/l, 10% heat inactivated fetal calf serum and 1% antibiotic solution (penicillin 10 000 IU/ml and streptomycin 10 000 U/ml).

Cytokines

Human γ-interferon, IL-10 and TGFβ$_1$ were purchased from Genzyme (Kent, UK). All cytokines were prepared as sterile, filtered solutions in culture media, and then stored frozen. Although specific bio-assays of these preparations were not performed for this series of experiments, all of these cytokine preparations demonstrated biological activity in cell culture systems used by other workers in our group.

Experimental incubations

For each experiment, HT29/19a cells were harvested from the stock cultures and added to separate 4.5 cm^2 wells in a 12-well culture plate. The cells were then incubated for 72 h. Each experiment was done in quadruplicate.

Experimental protocols

HLA-DR expression was induced by γ-interferon. Experiments were performed using concentrations of γ-interferon of 10 U/ml and 100 U/ml. The effects of IL-10 and TGFβ$_1$ were assessed over cytokine concentrations of 0, 1, 10 and 100 U/ml. Three series of experiment were performed. Firstly, the effect of IL-10 was examined. Secondly, the effect of TGFβ$_1$ was examined, and thirdly, the effect of IL-10 and TGFβ$_1$ in combination was examined. Within each experimental series, two further experiments were set up. In one set of cultures, cytokine was added at the same time as γ-interferon was added (co-incubation). In a separate set of cultures, cytokine was added 24 h prior to the addition of γ-interferon (pre-incubation).

A further series of experiments were performed to examine the hypothesis that mucosal lamina propria lymphocytes from uninflamed colonic mucosa may inhibit colonic epithelial cell expression of HLA-DR. Colonic mucosal lamina propria lymphocytes (LPL) were isolated from uninflamed colonic mucosa obtained from surgical resection specimens using a previously described protocol.[29] All tissue was obtained from normal appearing mucosa at least 5 cm from any macroscopic disease. The diagnoses of the patients included carcinoma (six patients) and ischaemic colitis (one patient). The isolated LPL were maintained at a concentration of 10^6 cells per ml in RPMI culture media supplemented with 10% heat inactivated fetal calf serum and 1% antibiotic solution (penicillin 10 000 IU/ml and streptomycin 10 000 U/ml). Following isolation, the LPL were maintained in culture at 37°C in an atmosphere of 5% CO_2 for 24 h. The cultures were then spun down at 750 × *g*, and the supernatants collected and frozen at –70°C. The LPL supernatants were thawed at 37°C and diluted with supplemented DMEM at a ratio of 1:2 (supernatant:DMEM). This mixture was then used to culture HT29/19a cells in a similar fashion to the cytokine experiments. The control cultures were maintained in a 1:2 mixture of supplemented RPMI and DMEM. Due to limited volumes of supernatant, this experiment was only done using γ-interferon of 100 U/ml. The composition of the supernatants was not evaluated.

Flow cytometry

At the end of the incubation period, the monolayers were disrupted and single cell suspensions created using EDTA. Cell viability was assessed by Trypan Blue staining, and any samples with viability was less than 90% were discarded. The cells were then stained with fluoroscein isothiocyanate (FITC)-conjugated anti-HLA-DR (Dako), a mouse anti-human monoclonal antibody to the β chain of HLA-DR. FITC-conjugated anti-IgG1 (Becton Dickinson) was used as the negative isotype control. The cells were fixed with 1% paraformaldehyde in PBS/0.1% sodium azide, and analysed within 2–4 days.

The samples were analysed using a Becton Dickinson flow cytometer using LYSIS II software. Fluorescence histograms for the anti-HLA-DR cells were generated for each sample. Five thousand cells per gate were counted. The data recorded included (a) the percentage of cells of each sample that showed fluorescence with the FITC-labelled anti-HLA-DR, and (b) the mean/median fluorescence intensity of the stained cells.

Statistical analysis

The data from each experimental series was analysed using a one-way analysis of variance using Excel® (Microsoft) software.

Results

Effect of γ-interferon

Cells were 70–80% confluent at the time of harvest. The percentage of cells staining positive for HLA-DR increased with increasing dose of γ-interferon, although no further increases were noted at concentrations of γ-interferon in excess of 50 U/ml. Within the population of cells staining positive for HLA-DR, there were no significant differences between median or mean fluorescence intensity with cells exposed to different doses of γ-interferon. The viability of cells in all experiments ranged from 92% to 95%, with no significant differences observed across any of the different interventions.

Some variation in the sensitivity of the cells to γ-interferon was noted from passage-to-passage. This occurred despite the use of identical reagents and culture protocols, and in the absence of infection. Similar variation has been seen by other workers using HT29 cell line.[12] In view of this observation, the direct comparison of results obtained from different passage generations is invalid. The cellular basis of this variability is unclear.

Effects of isolated IL-10 and TGFβ-1

Neither IL-10 nor TGFβ₁ in isolation, in the absence of γ-interferon, induced expression of HLA-DR in HT29/19a cells. Neither co-incubation nor pre-incubation with IL-10 resulted in any significant differences in the percentage of cells staining positive for HLA-DR at either concentration of γ-interferon (Fig. 1). Within the population of cells staining positive for HLA-DR, there were no significant differences between median or mean fluorescence intensity with cells exposed to different doses of IL-10 at either concentration of γ-interferon. In identical experiments, isolated TGFβ₁ had no effect on HLA-DR expression (Fig. 2).

FIG. 2. The effect of transforming growth factor γ-1 on γ-interferon induced HLA-DR expression. (The error bars represent ±1 standard deviation.) For all ANOVA, *P*-value >0.05.

Effects of combination IL-10 and TGFβ-1

When IL-10 and TGFβ₁ were added in combination in the absence of γ-interferon, no expression of HLA-DR was observed. In the presence of γ-interferon, the combination of IL-10 and TGFβ₁ significantly reduced the percentage of cells expressing HLA-DR in a dose dependent fashion (Fig. 3). The magnitude of the maximum reduction in percentage of cells expressing HLA-DR was greater in the groups incubated with the lower concentration of γ-interferon. Despite achieving statistical significance, the absolute reductions in expression of HLA-DR were small in each group (6–30%). The magnitude of the reductions in percentage of cells expressing HLA-DR was greater in the pre-incubation groups than in the co-incubation groups (for γ-interferon = 10 U/ml, 30% vs. 6%; for γ-interferon = 100 U/ml, 12.1% vs. 8.3%). Within the population of cells staining positive for HLA-DR, there were no significant differences between median or mean fluorescence intensity with cells exposed to different doses of IL-10 and TGFβ₁ at either concentration of γ-interferon.
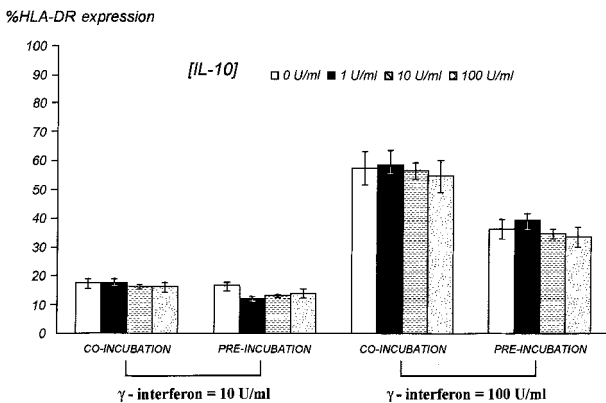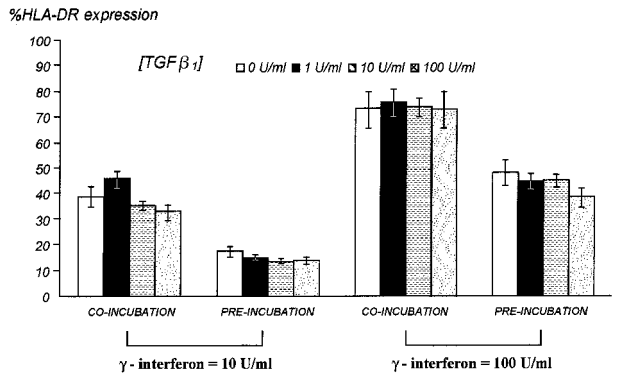

FIG. 1. The effect of interleukin-10 on γ-interferon induced HLA-DR expression. (The error bars represent ±1 standard deviation.) For all ANOVA, *P*-value >0.05.
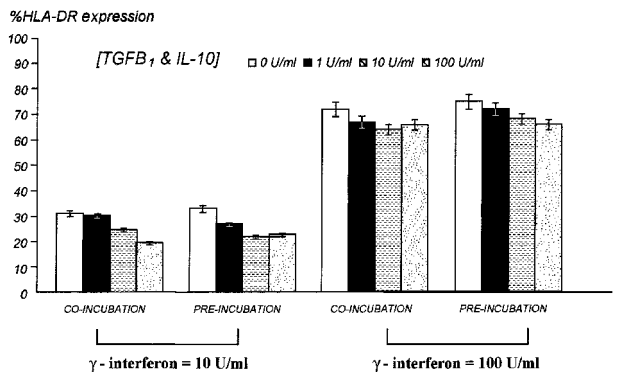

FIG. 3. The effect of combination interleukin-10 and transforming growth factor γ-1 on γ-interferon induced HLA-DR expression. (The error bars represent ±1 standard deviation.) *P*-value for ANOVA <0.05.

Effect of LPL supernatants on HT29/19A HLA-DR expression

No significant differences in HLA-DR expression were observed between cells cultured with LPL supernatant/DMEM culture medium and cells cultured with DMEM culture medium alone.

Discussion

On the basis of these results, neither TGFβ$_1$ nor IL-10 acting in isolation downregulate γ-interferon-induced expression of HLA-DR within this experimental system. There are no other published data examining the effect of IL-10 on expression of HLA-DR in a colonic epithelial cell line. Previous studies using TGFβ$_1$ have shown conflicting results. Darley *et al.*[11] demonstrated that TGFβ$_1$ inhibited induced expression of HLA-DR in a variety of cell lines. However, they found that nine different colorectal cell lines (including HT29) were resistant to the inhibitory effects of TGFβ$_1$ on both epithelial proliferation and epithelial expression of MHC molecules including HLA-DR. In contrast, Donnet-Hughes *et al.*[12] found that TGFβ$_2$ downregulated γ-interferon-induced HLA-DR expression by up to 75%. Several factors may explain the discrepancies between these different studies.

Firstly, the absence of inhibitory effects seen in the current study may reflect limitations of the experimental model. As Darley *et al.*[11] demonstrated, there is wide variation in the sensitivity of different cell lines to the effects of specific cytokines. It is interesting to note that even using the same cell line (HT29), different groups have generated diverse results. For example, our group has previously found that HT29 cells grown in glucose containing media do not show induction of HLA-DR on exposure to γ-interferon,[30] but this lack of responsiveness has not been found by other workers,[12] or in our experience with the current clone of HT29. These discrepancies may reflect differences in the degree of differentiation of the HT29 clones, as glucose influences the differentiation of these cells in culture.[25,27,30] Discrepancies between the results of different groups may represent subtle differences in the functional characteristics between the different clones of HT29 cells used.

A further confounding factor is that use of colorectal carcinoma cell lines is only an approximation of the functional behaviour of non-malignant cells. Recent work has shown some colorectal carcinoma cell lines, including HT29, have a mutation in the TGFβ receptor that renders them insensitive to the effects of TGFβ.[31] The ideal experimental model would be to use non-malignant epithelial cells. Whilst isolation protocols for human colonic epithelial cells are described,[7,32,33] it has been difficult to maintain non-malignant colonic epithelial cells in long-term cell culture.[34]

Methodological aspects of the experimental model may also have contributed to the failure to observe any inhibitory effects of isolated cytokines in the current work. One factor worth consideration is the incubation time used. Donnet-Hughes *et al.*[13] incubated cultures for 24–48 h and changed the cytokine containing media daily. In contrast, the cell cultures in our series were maintained for 72 h without any replenishment of the media. As cytokines are labile and have very short half lives *in vivo*, it is conceivable that the relatively long incubation time and the lack of replenishment of the cytokines may have reduced the sensitivity of the experimental system for detection of cytokine-mediated changes in HLA-DR expression.

In contrast to the effects of TGFβ$_1$ and IL-10 alone, small reductions in induced HLA-DR expression were observed when both cytokines were added in combination. The magnitude of the reductions in HLA-DR expression are relatively small compared with those observed by other workers.[13] As has been observed in a previous study,[12] the magnitude of the reduction in HLA-DR expression is greater when the induction signal is smaller (i.e. at lower doses of γ-interferon that are not within the saturated portion of the γ-interferon/HLA-DR expression dose response curve). Although the observed reductions in HLA-DR expression may represent a genuine biological effect, the absolute magnitude of the reduction in HLA-DR expression is small and may simply represent non-specific effects of having extra peptide in the culture media.

It is biologically plausible that TGFβ$_1$ and IL-10 acting in combination have much greater inhibitory effects on HLA-DR expression than either cytokine acting alone. It has been shown that TGFβ$_1$ and IL-10 act at different levels of cellular function. For example, IL-10 has been shown to downregulate macrophage TNF-α production by inhibiting production of TNF-α mRNA whereas TGFβ$_1$ inhibits macrophage TNF-α production by inhibiting TNF-α release.[35]

It is also possible that the small reductions in HLA-DR expression demonstrated in the current study have no relevance *in vivo*. Other workers have shown that the inhibitory effects of cytokines such as IL-10 are cell-type specific and also depend on the type of inducing signals used.[17] A major limitation of the current experimental model is that it ignores the interactions of other cell types found within in the mucosal microenvironment *in vivo*. It is possible that the anti-inflammatory properties of IL-10 and TGFβ$_1$ *in vivo* are mediated through other cell types such as macrophages and neutrophils,[14] and that epithelial cell HLA-DR expression is not the direct target of these cytokines *in vivo*. Even if the epithelial cells are not the direct targets of IL-10 and TGFβ$_1$, these cytokines may inhibit epithelial cell HLA-DR expression indirectly. For example, IL-10 is a potent inhibitor of monocyte production of γ-interferon,[36] which will in turn inhibit the HLA-DR expression by epithelial cells.

Finally, one can speculate that an 'off' signal for colonic epithelial HLA-DR expression is not necessary *in vivo* because of the kinetics of the colonic epithelial turnover. The colonic epithelium is constantly turning over with continuous loss of epithelial cells and replacement of these cells from proliferation in the colonic crypts. In this system, the loss of inflammatory signals such as γ-interferon maybe all that is necessary to result in the re-appearance of HLA-DR-negative epithelial cells. Our observation that lamina propria lymphocyte supernatants from uninflamed mucosa do not downregulate γ-interferon-induced HLA-DR expression is consistent with this hypothesis.

In summary, IL-10 and TGFβ$_1$ in combination, but not acting alone, directly downregulate γ-interferon-induced HLA-DR expression in colonic epithelial cells. The magnitude of these effects are small, and may reflect limitations of the specific experimental model. The biological relevance of these findings is unclear. Given the potential therapeutic significance of IL-10 and TGFβ$_1$ in controlling mucosal inflammation, further investigation is warranted.

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