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Transforming growth factor-β enhances interleukin-6 secretion by intestinal epithelial cells

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SUMMARY

Recent reports have suggested that transforming growth factor-β (TGF-β) may have an important role in IgA immune responses, e.g. induction of surface IgM$^+$ B cells to commit to IgA. TGF-β is also an important regulatory cytokine for the maturation of intestinal epithelial cells. Using the IEC-6 rat intestinal epithelial cell line as a model system, TGF-β1 was found to enhance interleukin-6 (IL-6) secretion by the IEC-6 cells. The IL-6 was produced in a dose-dependent manner and secretion could be specifically inhibited by an anti-TGF-β1 antibody. IL-6 production by the IEC-6 cells was confirmed by using a rabbit anti-mouse IL-6 antibody which completely neutralized the IL-6 present in the IEC-6 cell supernatant. The enhancement of IL-6 secretion was found to involve a low-level enhancement in the expression of RNA for IL-6. The induction of IL-6 secretion was also reversible when TGF-β was removed. These results suggest that the action of TGF-β on intestinal epithelial cells may play an important role in immune responses at the intestinal mucosa.

INTRODUCTION

Intestinal epithelial cells (IEC) have long been known to function as a mediator for transport of locally produced polymeric IgA into the lumen of the intestine.[1,2] Yet, recent studies have begun to indicate that IEC may have an expanded role in immunity at the intestinal mucosa. Intestinal epithelial cells express class II major histocompatibility complex (MHC) antigens[3,4] and are capable of presenting antigen to CD8$^+$ T cells in a rat model[5] and in humans.[6] Immunostaining of human colon has shown that intestinal epithelial cells can express interleukin-6 (IL-6)[7] and isolated IEC stimulated with lipopolysaccharide (LPS) have been shown to contain messenger RNA (mRNA) for both IL-1 and IL-6.[8] These two cytokines, which are produced by most antigen-presenting cells, help in B- and T-cell responses and induce hepatocytes to release acute phase proteins in inflammation.[9] However, little is known of the factors which induce IEC to produce these cytokines.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine produced by lymphocytes,[10,11] macrophages,[12] and other cells such as intestinal epithelial cell lines.[13–15] This cytokine inhibits the proliferation of many cell types including B cells,[10] T cells[11] and intestinal epithelial cells,[14] and has been shown to inhibit endothelial cell proliferation by arresting these cells in G1 phase.[16] TGF-β also inhibits IgG and IgM production by B cells, yet, in murine systems, TGF-β enhances the production of IgA[17,18] suggesting a role for TGF-β in mucosal IgA responses. This enhancement is due to the induction of sterile α-messenger RNA (mRNA) transcripts and subsequent switches to IgA in B cells stimulated with LPS.[19]

Intestinal epithelial cell lines not only secrete TGF-β but also have receptors for this cytokine.[20] This has added credence to a report which suggested that TGF-β may act as an autocrine regulatory factor for IEC. In this study, TGF-β not only inhibited the proliferation of a non-transformed rat IEC cell line (IEC-6)[21] but also induced these cells to exhibit sucrase activity, an indicator of IEC cell maturation.[22]

Since TGF-β has been shown to have maturational effects on IEC as well as having an important role in IgA B-cell commitment, we have used the IEC-6 cell line to study the effect of TGF-β on the production of IL-6 by IEC. In our study, TGF-β was found to induce a dose-dependent enhancement of IL-6 secretion by the IEC-6 cells. This production of IL-6 was determined to be reversible, since the removal of TGF-β resulted in a reduction of IL-6 secretion to near background levels.

MATERIALS AND METHODS

Cytokines and reagents

The cytokines used in this study were obtained as follows: porcine TGF-β1 was obtained from R & D Systems (Minneapolis, MN) and recombinant murine IL-6 was a kind gift from Dr F. Lee (DNAX Research Institute, Palo Alto, CA). An IgG fraction of rabbit anti-porcine TGF-β1 was obtained from R & D Systems and a purified normal rabbit IgG fraction was purchased from Sigma Chemical Co. (St Louis, MO).

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; IEC, intestinal epithelial cells; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; SC, secretory component; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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A rabbit anti-mouse IL-6 was a kind gift from Dr G. Fuller (University of Alabama at Birmingham, AL) and was prepared by immunizing a rabbit with an intradermal (i.d.) injection of 10 μg recombinant mouse (rm) IL-6 in complete Freund's adjuvant:incomplete Freund's adjuvant (CFA:IFA) (8:1) followed by 5 μg rmIL-6 in IFA on Days 10 and 20. Then at 15-day intervals, the rabbit received 10 μg rmIL-6 in IFA (i.d.) and bleedings were taken between Days 50 and 60. An IgG fraction was prepared from serum by precipitation of immunoglobulins in 50% ammonium sulphate and passage over a Protein G-agarose column (Boehringer Mannheim, Indianapolis, IN).

Cell culture

The IEC-6 cell line[21] was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) with 4·5 g/l glucose containing 5% foetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, UT), 0·1 IU/ml bovine insulin (Sigma), 2 mM L-glutamine, 80 μg/ml gentamicin, 25 IU/ml penicillin, and 25 μg/ml streptomycin (Mediatech, Washington, DC). The Hoechst 33258 staining method[23] was used to monitor the cells periodically for mycoplasma contamination. All experiments were performed on cells at the twentieth passage or less.

Assay for production of IL-6 by IEC

Culture supernatants from IEC-6 cells for measuring IL-6 production were derived as follows. IEC-6 cells at 2×10^5 cells/1·5 ml of tissue culture medium were added to 12-well tissue culture plates and incubated for 2 days. The medium was then removed and the cells were washed with serum-free medium prior to adding 1·5 ml/well of 1% FCS culture medium containing TGF-β1. At this time, each well contained approximately $2{\cdot}9 \times 10^5$ cells. After incubation for 1–4 days, the supernatants were collected, passed through a 0·45 μm filter, and frozen until assayed for IL-6 content. In some experiments, the cells were treated for 90 min with 1 μg/ml actinomycin D (Sigma) and washed three times prior to culture with TGF-β1.

The amount of IL-6 in each culture supernatant was determined by a proliferative assay using the IL-6-dependent, 7TD1 mouse–mouse hybridoma.[24] Briefly, 50 μl aliquots of serial twofold dilutions of culture supernatant or a rmIL-6 standard were prepared in a 96-well culture plate (Falcon, Becton Dickinson, Lincoln Park, NJ) with RPMI-1640 containing 10% FCS (Hyclone), 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES and antibiotics. Then 2×10^3 washed 7TD1 cells in 50 μl were added to each well. The cultures were incubated for 4 days at 37° and the resulting proliferation was measured using a colorimetric assay.[25] One unit of IL-6 was defined as the reciprocal of the dilution giving 50% maximal stimulation of proliferation. This assay routinely yielded a standard curve which was linear between 1 and 50 U/ml of IL-6.

The 7TD1 cells showed no proliferation in culture medium alone or in medium with TGF-β1 (0·1–10 ng/ml) alone as well as up to 200 U/ml of IL-1, IL-2, IL-4, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or 250 ng/ml of epidermal growth factor. However, the 7TD1 cells did proliferate slightly with IL-5 where 200 U/ml of IL-5 appeared as approximately 11 U/ml of IL-6. Also, TGF-β1 at 2 ng/ml was found to have no effect on the rmIL-6 stimulated proliferation of the 7TD1 cells. Higher concentrations of TGF-β1 were found to be slightly inhibitory, yet only with IL-6 levels below 6 U/ml.

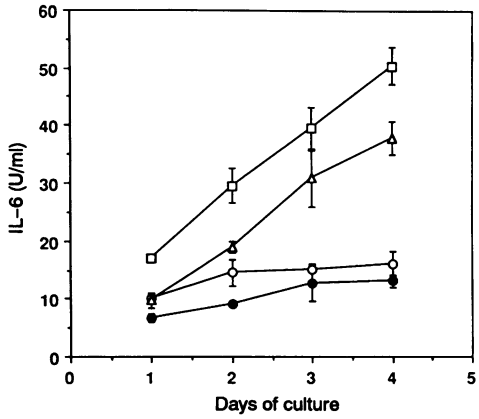

Figure 1. TGF-β1-induced enhancement of IL-6 secretion by cultured IEC-6 cells. IEC-6 cells (2×10^5 cells/well) were cultured for 2 days prior to adding 0 ng/ml (●), 0·2 ng/ml (○), 2 ng/ml (△), or 10 ng/ml (□) TGF-β1. Culture supernatants were then collected on the appropriate day after adding the TGF-β1 and the IL-6 content was determined by a proliferative assay with the 7TD1 hybridoma cell line.

The proliferation of the IEC-6 cells in 12-well culture plates prepared as above was determined by removing the culture supernatant and treating the adherent cells with 0·5% trypsin in 6 mM EDTA (Sigma) for 10 min at 37°. The cells were then diluted in medium with 10% FCS, pipetted vigorously to break up cell clumps and counted using a haemocytometer.

Dot blot analysis for cytoplasmic RNA content

The IEC-6 cells (1×10^6 cells/25 cm² flask) were cultured in DMEM with 5% FCS and supplements for 1 or 2 days then the medium was removed and DMEM with 1% FCS with or without 2 ng/ml TGF-β1 was added. At the appropriate time interval, the cells were removed by trypsin and EDTA treatment and washed once in medium containing 5% FCS followed by two washes in phosphate-buffered saline (PBS). Total cytoplasmic RNA was then isolated and diluted to amounts equal to between 5×10^5 and $2{\cdot}5 \times 10^4$ cells before binding onto nitrocellulose filters as previously described[26,27] using a dot blot apparatus (Schleicher & Schuell Inc., Keene, NH).

The RNA dot blots were hybridized overnight under previously described conditions[27] with ^{32}P-labelled cDNA for murine IL-6,[28] kindly provided by Dr F. Lee (DNAX). The murine IL-6 cDNA has previously been shown to be 92% identical to an isolated rat cDNA for IL-6.[29] The blots were then washed four times in 0·5–1 × SSC and 0·1% SDS at 42° before exposing to X-ray films with an intensifier screen at −70°. All dot blots were also probed with the cDNA for β-actin and/or rat cyclophilin for comparison of RNA content.

Statistics

Differences between the mean values were determined significant by ANOVA and Duncan's new multiple range test with the probability set at 0·05% or 0·01%.[30]

RESULTS

TGF-β induces IEC-6 cells to secrete IL-6

Figure 1 shows the result from a typical experiment where IEC-6 cells were cultured from 1 to 4 days with TGF-β1 at 0, 0·2, 2 or 10

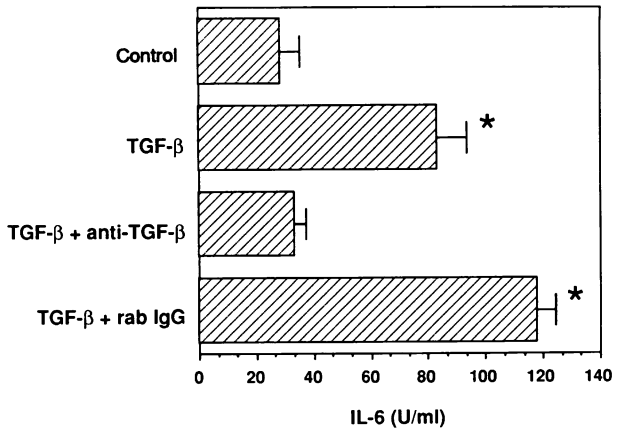

Figure 2. Anti-TGF-β1 antibody inhibits the TGF-β1-induced IL-6 secretion. IEC-6 cells were cultured with 2 ng/ml TGF-β1 as in Fig. 1 except that some cultures contained 100 μg/ml rabbit anti-TGF-β1 or 100 μg/ml normal rabbit IgG. Culture supernatants were collected on Day 4 and assayed for IL-6 content as in Fig. 1. Asterisk indicates a significant difference from the control values ($P < 0.01$).

ng/ml. The addition of 2 or 10 ng/ml TGF-β1 resulted in a significant enhancement of IL-6 secretion by the IEC-6 cells ($P < 0.01$) and the amount of IL-6 produced was dosage dependent. Figure 1 is representative of four separate experiments.

Previous reports have shown that TGF-β could inhibit the proliferation of IEC-6 cells in culture.[14,22,31] However, this phenomenon did not alter the finding that TGF-β1 enhanced IL-6 secretion by the IEC-6 cells since IL-6 levels adjusted for the total cells recovered at the time of harvest continued to show the same general trend as seen in Fig. 1 (e.g. 3.6 ± 0.3 and 17.3 ± 0.9 U IL-6/10^5 cells for untreated and 2 ng/ml TGF-β1 treated cultures, respectively, at Day 4).

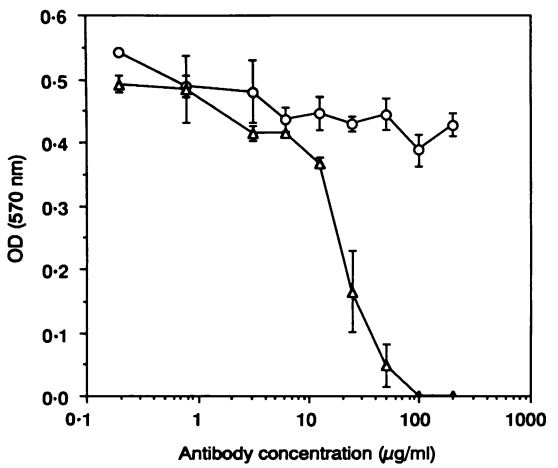

Figure 3. Polyclonal rabbit anti-mouse IL-6 antibody neutralizes IEC-6-derived IL-6. An IEC-6 supernatant from cells stimulated 4 days with 2 ng/ml TGF-β1 (containing 34 U/ml of IL-6) was incubated 45 min with dilutions of a polyclonal rabbit anti-mouse IL-6 antibody (△) or normal rabbit IgG (○) prior to adding 2×10^3 7TD1 cells/well. The proliferation of the 7TD1 cells after 4 days is shown as the OD at 570 nm from the colorimetric proliferation assay[25] minus the values from cells cultured without cytokines. This figure is representative of experiments with three separate supernatants.

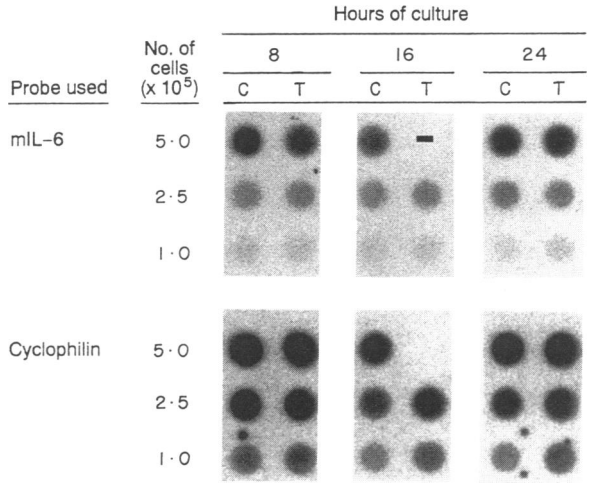

Figure 4. The effect of TGF-β1 treatment on the expression of mRNA for IL-6. IEC-6 cells (1×10^6 cells/culture) were cultured with or without TGF-β1 (2 ng/ml) for the indicated time. Total cytoplasmic RNA was isolated and the RNA corresponding to the number of cells indicated was fixed to a nitrocellulose membrane and probed for IL-6 or cyclophilin mRNA. C, control cells; T, TGF-β1-treated cells; (—) not done.

Table 1. The effect of pretreatment with actinomycin D on TGF-β1 induced IL-6 secretion by IEC-6 cells

Pretreatment*	Culture condition	IL-6 secreted (U/ml) Day 1	Day 2
None	Control	5.6 ± 1.0	14.1 ± 1.1
	TGF-β1	5.8 ± 0.3	23.4 ± 3.4
Actinomycin D	Control	13.2 ± 4.9	26.2 ± 5.6
	TGF-β1	14.8 ± 3.0	26.1 ± 1.1

* IEC-6 cells were pretreated for 90 min with 1 μg/ml actinomycin D and washed prior to culture with or without 2 ng/ml TGF-β1. Culture supernatants were then collected and IL-6 content was determined.

In another study, a polyclonal rabbit anti-TGF-β1 antibody was added along with the TGF-β1 cultures of IEC-6 cells (Fig. 2). The addition of this antibody resulted in a significant reduction in the amount of IL-6 produced by the TGF-β1 stimulated IEC-6 cells. By contrast, the addition of equal quantities of normal rabbit IgG resulted in a slight enhancement of IL-6 secretion by cells stimulated with TGF-β1. These results show that the IL-6 production by IEC-6 cells was due to the TGF-β1 and not a possible contaminant in the cytokine preparation.

The unstimulated control cultures did show some increase in IL-6 secretion over the 4-day interval which could not be accounted for by a correction for cellular proliferation. This IL-6 secretion was not due to the presence of endogenous TGF-β since unstimulated cultures with added anti-porcine TGF-β antibody showed no reduction in the baseline IL-6 secretion

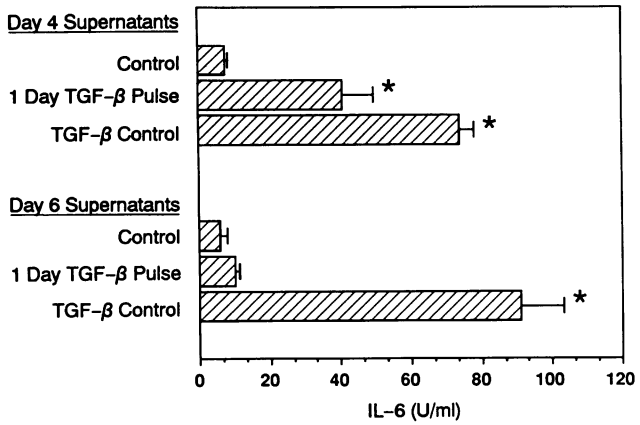

Figure 5. Effect of short-term incubation of IEC-6 cells with TGF-β1. IEC-6 cells were cultured with TGF-β1 (2 ng/ml) as in Fig. 1 except that the cells were washed on Day 1 and medium alone (Control and TGF-β 1 Day Pulse) or medium with 2 ng/ml TGF-β1 (TGF-β Control) was added. Culture supernatants were collected on Day 4 and the cells were again washed and incubated with medium alone or with 2 ng/ml TGF-β1 as above. On Day 6, supernatants were again collected and all supernatants were assayed for IL-6 content as in Fig. 1. Asterisk indicates a significant difference from Control cultures ($P < 0{\cdot}01$).

(data not shown). However, this does not rule out the possibility that some other factor in the FCS containing culture medium may have induced this low level of IL-6 secretion or that the antibody may not have neutralized the TGF-β produced by the rat IEC-6 cells.

Rabbit anti-mouse IL-6 antibody neutralizes the IL-6 produced by rat IEC-6 cells

A rabbit anti-mouse IL-6 antibody was used to determine whether the factor produced by the IEC-6 cells which stimulated 7TD1 cell proliferation was IL-6. Various dilutions of the rabbit anti-mouse IL-6 antibody were incubated with TGF-β1 stimulated supernatants from the IEC-6 cells prior to adding the 7TD1 cells (Fig. 3). Increasing concentrations of the anti-IL-6 antibody reduced in a dose-dependent way the proliferative capability of the 7TD1 cells until the addition of 100 μg/ml of anti-mouse IL-6 antibody completely inhibited the proliferation of the 7TD1 cells. This result clearly indicates that the antibody completely neutralized the IL-6 present in the IEC-6 cell supernatant. However, normal rabbit IgG added in the same quantities had no effect on the proliferation of the 7TD1 cells.

The effect of TGF-β on RNA synthesis

The effect of TGF-β on the expression of IL-6 mRNA was next determined by dot blot analysis. Results from these experiments showed no significant enhancement of RNA levels for IL-6 over that of untreated cells for any time interval tested (2–24, 48 and 72 hr; Fig. 4). However, pretreatment of the IEC-6 cells with the RNA synthesis inhibitor actinomycin D prior to culture with TGF-β1 showed that RNA synthesis was necessary for the enhancement of IL-6 secretion only after 2 days of culture (Table 1), and the pretreatment did not completely inhibit all IL-6 secretion confirming that the IEC-6 cells were already producing mRNA for IL-6. Actinomycin D pretreatment also seemed to enhance IL-6 secretion by the control cells suggesting that IL-6 secretion by the IEC-6 cells may normally be suppressed by a mechanism which requires active RNA synthesis. The actinomycin D pretreatment did not simply induce maximal IL-6 secretion by the IEC-6 cells since cultures containing recombinant human IL-1β alone or with the inhibitor yielded IL-6 levels after 2 days which were greater than three- and nine-fold higher, respectively, than similar cultures with TGF-β1 (data not shown). Therefore, the cells presumably still had the potential to produce even higher levels of IL-6 than shown in Table 1.

Short-term incubation of IEC-6 cells with TGF-β1 induces a transient secretion of IL-6

Experiments were performed to determine if TGF-β1 must be continually present in order to maintain IL-6 secretion by the IEC-6 cells. The IEC-6 cells were incubated as before with TGF-β1 except that on Day 1 after addition of the TGF-β1, the medium was removed and the adherent cells were washed twice with incomplete medium prior to addition of fresh medium alone (1 Day TGF-β Pulse cultures). Cytokine-free negative control cultures were also washed before adding fresh medium as were TGF-β1-containing positive control cultures which received medium with 2 ng/ml TGF-β1. On Day 4, the culture supernatants were collected and the cells washed twice again before fresh medium alone (for Control and 1 Day TGF-β Pulse cultures) or medium containing 2 ng/ml TGF-β1 (for TGF-β Control cultures) was added to the cultures. Culture supernatants were once again collected on Day 6. Figure 5 shows a representative result from three such experiments. In Day 4 supernatants, a 1-day pulse of TGF-β1 was sufficient to induce a significant enhancement of IL-6, yet the levels generally remained significantly lower than those in cultures which contained TGF-β throughout the incubation period ($P < 0{\cdot}01$ for this experiment). However, 1 Day TGF-β Pulse culture supernatants collected from the final 2 days of a 6-day culture (Day 6 Supernatants) yielded reduced IL-6 levels near to those of Control culture supernatants. This result shows that the TGF-β induced production of IL-6 is reversible, and maximum production of this cytokine occurs in TGF-β-treated IEC-6 cultures.

DISCUSSION

Studies of immune responses at mucosal surfaces have focused mainly on the interactions of accessory cells with T and B cells found at these sites. Recently, the potentially significant function of IEC in various aspects of mucosal immunity has gained wide attention. IEC are capable of presenting antigen to $CD8^+$ T cells[5,6] as well as producing cytokines important in T-cell responses.[7,8] However, little is known of the factors which influence these IEC functions.

In this report, we have presented evidence that IEC, as exemplified by the IL-6 cell line, can be induced to secrete enhanced levels of IL-6 by TGF-β1. That IL-6 was in fact produced was supported by the observation that supernatants from TGF-β1 stimulated IEC-6 cells supported the proliferation of the IL-6-dependent 7TD1 mouse B-cell hybridoma. To confirm the production of IL-6, a cross-reacting rabbit anti-mouse IL-6 antibody was used which efficiently neutralized the IL-6. A polyclonal rabbit anti-TGF-β1 antibody was also used to confirm that the added TGF-β1 was responsible for the

induction of IL-6 secretion. Also, the presence of TGF-β1 was required for sustained secretion of IL-6 since the IL-6 levels in supernatants of 1 Day TGF-β1 Pulse cultures returned to control levels after 6 days when the TGF-β1 was removed, indicating that this response was reversible.

An analysis of RNA expression suggested that the unstimulated IEC-6 cells were already producing mRNA for IL-6 and that IL-6 RNA levels were essentially unchanged after stimulation with TGF-β1. Experiments with actinomycin D pretreated cells confirmed that *de novo* synthesis of IL-6 mRNA was not necessary for IL-6 secretion; however, RNA synthesis was necessary for the enhancement of IL-6 secretion after TGF-β1 treatment. The resulting increase in RNA synthesis after TGF-β1 treatment, which yields an approximate threefold increase in IL-6 secretion, was probably a change which could not be easily quantitated.

Investigations into the functions of TGF-β have suggested that this cytokine has a role in the inflammatory response. It has been proposed that TGF-β released by platelets and other cells at wound sites induces cellular infiltration and initiates wound healing.[32,33] TGF-β has also been shown to induce IL-1 gene expression in monocytes[34] and peritoneal macrophages[33] as well as induce peripheral blood mononuclear cells,[35] articular chondrocytes[36] and pulmonary fibroblasts[37] to secrete IL-6. IL-6, like IL-1 and TNF-α, is an important cytokine in the inflammatory response which can induce macrophage differentiation and acute phase protein synthesis.[9] The fact that TGF-β1 induced IEC-6 cells to secrete IL-6 suggests that inflammatory responses at the intestinal mucosa may include a TGF-β mediated increase in IL-6 secretion by the IEC. Down-regulation of the IL-6 secretion after TGF-β removal also conforms to the mechanism of a short-term inflammatory response.

Several recent reports have demonstrated the effect of other inflammatory cytokines on IEC. Both IFN-γ[38] and TNF-α[39] were capable of enhancing the expression of the polymeric Ig receptor, secretory component (SC, on a human colon adenocarcinoma cell line (HT-29). IFN-γ also enhanced class II MHC antigen expression on these cells.[38] Furthermore, a mixture of IFN-γ and IL-4 was shown to enhance SC expression by HT-29 cells to levels greater than with either cytokine alone.[40] We have recently found that TGF-β could also enhance SC expression on the IEC-6 cells[31] again suggesting a role for TGF-β in immune responses which involve IEC.

A variety of intestinal epithelial carcinoma cell lines and the IEC-6 cell line can secrete TGF-β[15,41] and isolated villus IEC express TGF-β1 mRNA.[14] This, along with another study which showed that added TGF-β induced the expression of sucrase activity in the IEC-6 cell line,[22] which is believed to be an immature crypt epithelial type cell,[21] suggests that TGF-β may be an autocrine regulatory factor for the maturation of IEC. If IL-6 secretion by IEC could be induced by TGF-β, then the secretion of IL-6 may also be the normal status of mature IEC instead of being exclusively the result of an inflammatory response. However, the addition of anti-TGF-β1 antibody to unstimulated control cultures had no effect on the low-level secretion of IL-6 by the IEC-6 cells. This suggests that the amount of TGF-β produced by the IL-6 cells was of insufficient quantity to alter IL-6 secretion by these cells but this may not accurately reflect the conditions *in vivo*. In support of the hypothesis that IL-6 secretion may be the normal status of IEC, normal human small intestine stained with antibodies to IL-6 has shown that epithelial cells secrete IL-6, presumably in a non-inflamed situation.[7] Also, we have recently found that the IL-6 levels secreted by IEC-6 cells stimulated with IL-1 could be up to 10-fold higher than levels induced by TGF-β alone (D. W. McGee, K. W. Beagley, W. A. Aicher and J. R. McGhee, manuscript in preparation) indicating that IL-6 secretion induced by TGF-β may be of more importance in a non-inflamed situation.

The IL-6 produced by the IEC could have a number of possible functions in addition to its role in the inflammatory response. As mentioned before, IL-6 secreted by an IEC could help in T-cell responses if the IEC is acting as an antigen-presenting cell.[8] Also, IL-6 alone[42] or as a co-factor with IL-5[43] has been shown to preferentially enhance IgA secretion by Peyer's patch B cells. In this respect, the IEC may be of importance in maintaining an environment which helps IgA-committed B cells to differentiate terminally in intestinal tissues. Yet, the possibility also exists that the IL-6 may have some unknown autocrine function on the IEC itself such as growth enhancement or even to prolong the viability of the mature IEC. Indeed, a recent report has indicated that IEC may express receptors for IL-6.[7] These and other aspects of the effect of TGF-β on IEC are now being addressed by this laboratory.

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