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Growth Stimulation of a Human Colorectal Carcinoma Cell Line by Interleukin-1 and -6 and Antagonistic Effects of Transforming Growth Factor β1

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We analysed the effect of interleukin-1 (IL-1), IL-6 and transforming growth factor β1 (TGFβ1) on the growth of a panel of eight colorectal carcinoma cell lines. IL-1 stimulated growth of two lines (LS411N and LS1034) up to 20-fold and IL-6 enhanced proliferation of LS1034 more than 5-fold. Both cytokines also augmented colony formation of LS1034 in methylcellulose. Under both growth conditions IL-1 was the most potent stimulator. However, the addition of IL-6 to IL-1 synergistically enhanced proliferation of LS1034 in monolayer culture and additively augmented the number of colonies formed in methylcellulose. Furthermore, TGFβ1 strongly reduced the growth rate of LS1034. Low amounts of TGFβ1 markedly inhibited the response of LS1034 to IL-1 and totally abrogated proliferation induced by IL-6. We conclude that different cytokines can provide distinct signals for the regulation of growth of colorectal carcinoma cells.


INTRODUCTION

The term cytokines covers a number of soluble mediators that deliver signals from one cell to another. Within this group, the interleukins (IL) represent an important family. The spectrum of target cells affected by a single interleukin is generally rather broad. This is particularly true for IL-1 and IL-6, both molecules exerting activities on a large variety of different cell types.

Peripheral blood monocytes were originally described as the producer cells of IL-1. Evidence has accumulated, however, that numerous other cell types are also capable of secreting IL-1[1]. The effects of IL-1 on tumour cell growth are controversial. For some tumours IL-1 is cytotoxic [2] or cytostatic [3]. In contrast, IL-1 enhances the proliferation of several tumour cell lines [4–6] including one of colorectal origin [7]. The activities of IL-6, originally described as a B cell differentiation factor are also pleiotropic. IL-6 is produced by various tumours and
tumour cell lines [8–10]. In addition, IL-6 is implicated in the growth regulation of several malignancies, either in an autocrine [11, 12] or paracrine [13] fashion.

For transforming growth factor β (TGFβ) inhibitory effects on cell growth have been described for both neoplastic and non-neoplastic cells. However, TGFβ-sensitivity does not correlate with the presence of TGFβ-receptors, which are ubiquitously expressed [14]. Some tumour cells are markedly inhibited by TGFβ [15], while others are refractory though expressing functional receptors [16]. In addition, one fundamental mechanism behind the antiproliferative effect of TGFβ is its ability to antagonize the mitogenic effect of other growth factors. Such inhibitory properties of TGFβ have been reported for proliferative responses induced by several other cytokines [17, 18].

We have investigated the effects of IL-1, IL-6 and TGFβ on the growth of colorectal carcinoma cell lines, alone and in combination. We show, that IL-1 and IL-6 promote growth of colorectal carcinomas, but are more effective when acting in co-stimulation. In addition, we provide evidence that TGFβ, effectively abrogates both responses.

MATERIALS AND METHODS

Culture medium

Cell lines were cultured in a 1:1 mixture of Dulbecco’s modified eagle medium (DMEM) and nutrient mixture Ham’s F-12 (both Gibco) supplemented with HEPES (10mmol/l final concentration), L-glutamine (1.4 mmol/l final concentration), penicillin (100 U/ml) and streptomycin (100 μg/ml). This medium is referred to as EF medium.

Cell lines and culture conditions

We have used a panel of eight human colorectal cancer cell lines derived from primary tumours: Co-115, HT-29 (ATCC: HTB38), SW480 (ATCC:CCL228), WiDr (ATCC: CCL218) and Ls Isp-1 (obtained from Dr D. Lopez, Ludwig-Institute for Cancer Research, Sao Paulo, Brazil). The cell lines LS411N, LS513 and LS1034 have been established in our laboratory. LS411N was established from a biopsy specimen of a poorly differentiated caecal carcinoma, LS513 from a mucin-secreting tumour located at the Bauhin valve and LS1034 from a moderately to poorly differentiated caecal carcinoma. LS411N represents a tumour of Duke’s stage B, while LS513 and LS1034 were classified as Duke’s C. All cell lines were cultured in EF medium with 5% FCS (Seromed, Berlin). All cell lines were consistently found to be free of mycoplasma contamination using standard culture procedures (Mycop Test, Gibco).

Cytokines and reagents

Recombinant human (rhu) IL-1β was obtained from Biogen (Geneva), rhu IL-6 from Boehringer Mannheim (Bern) and TGFβ, from porcine platelets from R & D Systems (Minneapolis). [Methyl-3H]Thymidine (3H-TdR) was purchased from Amersham with a specific activity of 925 GBq/mmol.

Proliferation assay

Proliferation was assessed in EF medium. Cells were washed with PBS and cell suspensions were obtained by trypsinising monolayer cultures with 0.05% trypsin/0.02% EDTA (Seromed). They were washed with EF medium, resuspended in EF medium and distributed at 1 x 10^4 cells/well into 96-well flat-bottomed microtitre plates (Nunc) in a final volume of 200 μl EF medium. Cells were incubated for 5 days at 37°C and 5% CO2 in the presence or absence of cytokines. To assess the inhibitory effect of TGFβ on cytokine-induced proliferation, TGFβ, (0.5 ng/ml) was added to serial dilutions of IL-1 and IL-6 at the onset of the experiment. All samples were measured in triplicate. Proliferation was determined by measuring 3H-TdR incorporation after pulsing the cells with 3H-TdR (18.5 KBq/well) for the last 8 h of culture. Cells were harvested onto filter papers. The filters were dried and radioactivity was determined using a liquid scintillation counter.

Inhibition assay

Cells were distributed at 5 x 10^3 cells/well into 96-well flat-bottomed microtitre plates in a final volume of 200 μl of EF medium containing 5% FCS in the presence or absence of TGFβ. All samples were measured in triplicate. Proliferation was assessed by measuring 3H-TdR incorporation after pulsing the cells with 3H-TdR (18.5 KBq/well) for the last 8 h of a 4-day culture period.

Methylcellulose assay

Anchorage-independent growth was examined in a methylcellulose-based clonogenic assay [19]. Briefly, cells were suspended in EF medium supplemented with 0.9% methylcellulose (Fluka, Buchs, Switzerland). 1 ml aliquots were plated into bacteriological petri dishes (35 mm diameter, Greiner, Nürtingen, Germany) at a final cell concentration of 1 x 10^4 cells/plate. Cytokines were added directly to the plates. Cells were incubated at 37°C in a fully humidified atmosphere of 5% CO2, 5% O2 and 90% N2. Colonies of more than 50 cells were counted after 2 weeks under an inverted microscope. All samples were set up in triplicate plates.

Statistical analysis

Significance of differences between responses to growth factors and untreated control cells was calculated using the Student’s t-test.

RESULTS

IL-1 and IL-6 stimulate proliferation of colorectal carcinomas in monolayer culture

We tested IL-1 and IL-6 for growth stimulation on eight human colorectal carcinoma cell lines. Growth of two cell lines, LS411N and LS1034, was enhanced by IL-1. In addition, LS1034 was also stimulated by IL-6. Figure 1 shows representative dose–response curves for both cell lines. IL-1 proved to be a very potent stimulator of LS1034 cells and enhanced growth up to 20-fold (Fig. 1a). IL-6 induced a lower response, but enhanced proliferation of LS1034 nearly 6-fold (Fig. 1a). The effect of IL-1 on growth of LS411N was less pronounced. IL-1 stimulated growth above 2-fold in a dose-dependent way, while IL-6 was not effective over the whole dose range tested (Fig 1b).

A single addition of IL-1 (20 ng/ml) or IL-6 (100 U/ml) on day 0 stimulated cell division of LS1034 up to 14 days. This effect was significant from day 5 on (P < 0.05). By day 14, the
number of LS1034 cells cultured with IL-1 or IL-6 was enhanced by a factor of 6.2 and 3.0, respectively (data not shown).

**IL-1 and IL-6 stimulate anchorage-independent colony-formation**

We used the same conditions to investigate the effect of IL-1 and IL-6 on anchorage-independent colony-formation in methylcellulose. Both cytokines significantly increased the number of colonies of LS1034. In agreement with our results obtained in monolayer culture, IL-1 was more effective than IL-6 and enhanced colony-number more than 3-fold (Table 1). With IL-6 colony numbers were enhanced up to 180% with 200 U/ml (Table 1 and data not shown). LS411N cells did not form colonies under these conditions, even in the presence of IL-1 (data not shown).

**IL-1 and IL-6 cooperate synergistically in stimulating cell proliferation**

We next investigated the response to the concomitant addition of IL-1 and IL-6. For co-stimulation experiments we used 100 U/ml of IL-6, which per se induced significant proliferation ($P<0.0001$) (Fig. 2). However, in combination with IL-1 the proliferation of LS1034 was further enhanced as compared to the response obtained with IL-1 alone (Fig. 2). In fact, the dose–response curve was shifted to the left about 3-fold and maximal stimulation occurred at a higher plateau. The effect of IL-6 was most pronounced at the lowest doses of IL-1 tested. With IL-1-concentrations of 10 ng/ml or below the response upon simultaneous addition of IL-6 was synergistic ($P<0.005$) and potentiated about 5-fold. On the other hand, IL-6, which did not stimulate growth of LS411N cells, failed to augment IL-1-induced proliferation of this cell line (data not shown).

We also determined the effect of a combination of IL-1 and IL-6 on colony-formation of LS1034 in methylcellulose. IL-6 alone induced a small increase in colony number and further augmented the number of colonies obtained with IL-1 alone (Table 1). However, the effect of the combination of both factors was less pronounced than in monolayer cultures. Upon

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**Table 1. Induction of colony-formation by co-stimulation with IL-1 and IL-6**

<table>
<thead>
<tr>
<th>IL-1 (ng/ml)</th>
<th>Number of colonies (S.D.)*</th>
<th>Medium</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>351 (44)</td>
<td>460 (39)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>263 (56)</td>
<td>308 (44)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>185 (41)</td>
<td>254 (22)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>184 (23)</td>
<td>196 (10)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>97 (20)</td>
<td>143 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*LS1034 cells were cultured in the presence of rhu IL-1B as indicated together with medium or rhu IL-6 (100 U/ml). Colonies were counted after 2 weeks. Values represent the mean of triplicate plates.

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**Fig. 2. IL-6 enhances IL-1 induced proliferation.** LS1034 cells were cultured in the presence of serial dilutions of rhu IL-1B alone (C), rhu IL-6 alone (100 U/ml, □) or IL-1 and IL-6 (O). Values represent the mean of triplicate wells (S.D.). $^3$H-TdR uptake in the presence of culture medium was 1.738 (433) cpm.
Fig. 3. TGFB_1 inhibits growth of LS1034. Cells were cultured in the presence of serial dilutions of TGFB_1. Values represent the mean of triplicate wells (S.D.) (as in Legend to Figs 1 and 2). 3H-TdR uptake in the presence of culture medium was 9.157 (±0.45)cpm.

simultaneous addition of both cytokines the number of colonies was enhanced additively (Table 1).

**TGFB_1 inhibits proliferation of LS1034**

Since TGFB inhibits growth of several tumours including colon carcinomas [15] we tested TGFB_1 for growth inhibitory effects on LS1034 and LS411N cells. Growth of LS411N was not affected (data not shown). In contrast, TGFB_1 strongly inhibited growth of LS1034 in a dose-dependent way. At 1 ng/ml the proliferation was almost completely abrogated and even concentrations below 0.1 ng/ml reduced growth significantly (P < 0.01) (Fig. 3). The cyto-inhibitory effect was completely neutralised by anti-TGFB antibodies (data not shown).

**TGFB_1 abrogates IL-1- and IL-6-induced cell proliferation**

TGFB_1 effectively antagonises proliferative responses induced by several cytokines [17, 18]. Thus, we examined whether TGFB_1 would influence proliferation of LS1034 induced by IL-1 and IL-6. TGFB_1 was indeed an efficient inhibitor of both responses (P < 0.01). In the presence of TGFB_1, the response to IL-1 up to a concentration of 10 ng/ml was completely blocked. With higher doses the proliferation was strongly inhibited (> 78%). However, IL-1 still induced slight proliferative effects (P < 0.0001) at these concentrations (Fig. 4a). Likewise, TGFB_1 totally abrogated the response to IL-6 and even the highest concentration of IL-6 (200 U/ml) did not elicit any response exceeding the proliferation rate of cells incubated with culture medium alone (Fig. 4b).

**DISCUSSION**

We report growth stimulation of colorectal carcinoma cell lines by IL-1 and IL-6. The LS1034 cell line provides evidence that both cytokines can act together to support growth of such tumours. IL-1 has been found to be mitogenic for numerous tumour cell lines [4-6] and the same is true for IL-6 [11, 12]. However, growth stimulation of colorectal carcinomas by IL-1 has only been observed with few cell lines [7, 20], and growth stimulation by IL-6 has not been reported so far.

IL-1 and IL-6 display as well antiproliferative effects on tumour cell growth. IL-1 is directly cytotoxic for melanomas [2] and inhibits growth of malignant mammary cell lines [21]. Growth arrest in G1 phase has been attributed to IL-1 and IL-6 [22]. We have shown that colorectal carcinoma cells were driven through the whole cell cycle by IL-1 and IL-6. They stimulated DNA synthesis and also increased cell number. Thus, the effects of these cytokines on the proliferation of tumour cells appear to vary with the cell type.

The biological activities of IL-1 and IL-6 partially overlap. Both factors cooperatively influence cell growth, either in stimulating proliferation [23] or in reducing cell growth [24]. The combination of IL-1 and IL-6 was an effective stimulus for the proliferation of colorectal carcinomas. We have recorded synergistic effects of LS1034 cells by measuring thymidine uptake, but only additive enhancement of colony formation. Indeed, the two systems measure different parameters. The methylcellulose assay reflects the number of cells which have originally started dividing to give rise to a colony. In this context it is of note that the magnitude of colonies growing in the presence of IL-1 exceeded the size of those colonies growing in the presence of medium. In contrast, in the proliferation assay measuring thymidine uptake, every growing cell scores positive. The intermediate values of the stimulation index obtained by counting viable cells is in good agreement with this.

Solid tumours are frequently infiltrated in vivo by macrophages and lymphocytes. Once activated, these cells secrete several cytokines which contribute to the destruction of tumour cells, among those IL-1 and IL-6. Provided that growth of tumour cells is stimulated by these factors, as shown for LS411N cells, enhancement of this effect is to be expected.
and LS1034 cells, the production of IL-1 and IL-6 at the tumour site may be deleterious to the host. Certain tumour cells may gain a selective growth advantage, thereby escaping the surveillance of the host immune system. IL-1 appears to be only exceptionally expressed in host cells infiltrating primary colorectal tumours, while mRNA for tumour necrosis factor α (TNFα) has been frequently detected [25]. Since TNFα is a potent inducer of IL-1 and IL-6, it may, therefore, create a favourable microenvironment to promote tumour cell growth. It is of interest that the combination of IL-1 and IL-6 was most effective at low cytokine-concentrations, which are probably closest to the in vivo situation.

IL-6 functions as an autocrine growth factor in several types of malignancies [11,12] and its potential role as an autocrine growth factor has been suggested for colon carcinomas [26]. In addition, IL-1 is a potent inducer of IL-6 [27]. Therefore, it is tempting to speculate that IL-6 produced by colorectal carcinoma cells themselves may contribute to growth stimulation. However, we have not obtained evidence that autocrine IL-6 contributes to proliferation of LS1034 under the culture conditions used: (a) we have performed experiments in which we delayed the addition of exogenous IL-6. The synergistic response with IL-1 was only seen if IL-6 was present throughout the experiment. A delay of 24 h caused a rapid decrease of proliferation (data not shown). (b) The addition of IL-6 shifted the dose-response curve of IL-1 to a higher plateau, indicating the participation of another receptor. If endogenous IL-6 were already present, maximal proliferation in response to IL-1/IL-6 could have been equal only. (c) Anti-IL-6 Ab did not affect proliferation of LS1034 cells (data not shown). Still, we cannot totally exclude that LS1034 cells may secrete very low levels of IL-6.

Different mechanisms account for anti-proliferative effects mediated by cytokines. IL-1 and TNFα are directly cytotoxic for tumour cells [2,22]. Though TGFβ inhibits numerous proliferative responses, no direct cytotoxic effects of this molecule have been reported. We also did not observe cytotoxic effects of TGFβ1 on LS1034 cells (data not shown). However, we observed a change in morphology of LS1034 cells after exposure to TGFβ1. They exhibited a more flattened phenotype and grew in more uniform monolayers than untreated control cells. Similar effects have been observed with another human colorectal carcinoma [15] and endometrial carcinoma cell lines [28]. Such changes in cell morphology induced by TGFβ1 are accompanied by differentiative events [15]. In addition, TGFβ1 induced differentiation of colorectal tumours [29], which in turn may account for the antagonistic effect of TGFβ1 on the proliferation induced by IL-1 and IL-6.

This differentiation may indeed be reversible. In the presence of TGFβ1, colonies of LS1034 developed at a very low frequency (approximately 7 × 10−4) in methylcellulose. However, upon restesting all were sensitive to the cytotoxic-inhibitory effect of TGFβ1 (data not shown). Thus, upon removing TGFβ1, the cells obviously regained a state of susceptibility to this factor. The same may apply for responsiveness to IL-1 and IL-6. Of course, TGFβ1 might work via other mechanisms, such as modulation of receptors and intracellular targets for other growth factors. Multiresponsive lines such as LS1034 may, therefore, provide a useful tool to elucidate these events in further detail.

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Differential Expression of Insulin-like Growth Factor Binding Proteins in Human Non-small Cell Lung Cancer Cell Lines

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The possible expression and secretion of insulin-like growth factor binding proteins (IGFBPs) by non-small cell lung cancer (NSCLC) cell lines was investigated and compared with possible IGFBP expression by primary NSCLC tumours. Cells growing under serum-free conditions released binding proteins with apparent molecular masses of 26-43 kD when analysed by a ligand blotting method under non-reducing conditions. Additionally, northern blot analysis of total RNA from NSCLC cell lines and tumours was performed using cDNAs coding for each of IGFBP-1, IGFBP-2, and IGFBP-3. This analysis revealed expression of all three mRNAs to varying degrees by all cell lines. In contrast, all primary tumours analysed expressed predominantly IGFBP-2 and IGFBP-3 and none showed any evident expression of IGFBP-1. Both NSCLC cell lines and tumours synthesise IGFBPs but the pattern of expression differs significantly between cell lines and primary tumours.


INTRODUCTION

INSULIN-LIKE GROWTH FACTORS (IGFs) are peptides which modulate growth in a variety of tissues and cell types [1]. IGFs are present in the circulation, in tissues, and in cell culture media and in all these locations are found to be bound to specific binding proteins [2]. These IGF-binding proteins (IGFBPs) bind both IGF-I and IGF-II with high affinity and specificity, and do not bind insulin [3]. There are at least four distinct forms of human IGFBPs which differ in terms of molecular mass, binding specificities and distribution in biological fluids and which have been cloned: IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4.

IGFBP-1 is the predominant IGFBP in amniotic fluid and IGFBP-1 mRNA is detected in decidua and secretory endometrium [4]. Elsewhere IGFBP-1 mRNA is found only in the liver (and in the hepatic HEP G2 cells) with concentrations higher in fotal compared to adult liver [5]. Analysis of mRNA in various tissues and cell lines reveals a different pattern of expression for IGFBP-1 and IGFBP-2. IGFBP-2 mRNA is detected in adult liver, brain, in Jurkat and kidney 293 cells, but not in HeLa, Namalwa and HEPG2 cells [6]. IGFBP-1 and IGFBP-2 are proteins with 40% sequence identity [6]. Both proteins contain regions of clustered Pro, Glu and Thr residues (PEST regions), a Arg-Gly-Asp sequence (RGD motif) and a cystein-rich amino-terminus [3, 6]. The major IGF-BP in human adult serum is a 125-150 kD complex which dissociates under acidic conditions, releasing free IGFs and an acid stable IGFBP subunit. This binding subunit is referred to as IGFBP-3 (formerly IGFBP-53) with an apparent molecular mass of 53 kD under non-reduced and 43 kD under reduced conditions. Sequencing of cloned IGFBP-3 cDNA reveals a cysteine-rich primary structure of 264 residues and a predicted molecular mass of 28.7 kD [7]. The sequence has a 33% aminoacid identity including conservation of all 18 cysteine residues with IGFBP-1. Recently two additional IGFBPs have been described. One in conditioned medium from human bone cells and has limited aminoterminal sequence identity with IGFBP-1, IGFBP-2 and IGFBP-3 and appears to inhibit the action of IGF [8]. The other is a binding protein found in cerebrospinal fluid which specifically binds IGF-2 [9].

The biological functions of these binding proteins remain