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TRANSFORMING GROWTH FACTOR β_2 UP-REGULATES GM-CSF GENE IN HUMAN BLADDER CARCINOMA CELL LINE HTB 5637

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ABSTRACT

Transforming growth factor betas are multifunctional polypeptides in the cytokine superfamily. They have a growth inhibitory role on hemopoietic progenitor cells in semisolid colony assay as well as in long-term bone-marrow culture. TGF-β_2 represses stromal cells, stem cell factor gene transcription, and decreases the stability of c-kit transcripts in hemopoietic cells. TGF-β also modulates GM-CSF production from human lymphocytes. The present study reveals the TGF-β_2 role in production of GM-CSF in HTB 5637, human bladder carcinoma cell line. HTB 5637 cells were treated with 5 ng/mL of human TGF-β_2, viable cells were counted and GM-CSF concentration was determined. No antiproliferate activity of TGF-β_2 on HTB 5637 cell line was observed. Biological assay showed increased levels of GM-CSF in the supernatant of cultured cells. However this increase was lower than that expected from ELISA. Since TGF-β may be an active suppressor factor regulating hemopoiesis, it seems that some inhibitory factor(s) may be produced (increased) in response to TGF-β_2 treatment. It has been shown that GM-CSF mRNA content from HTB 5637 cell line is very stable and this stabilization is translational dependent. Using Slot blot and Northern blot analysis, we determined that TGF-β_2 upregulated GM-CSF gene expression in HTB 5637 cell line. The results suggest that TGF-β_2 upregulates the production of GM-CSF gene at the transcriptional level.

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INTRODUCTION

Transforming growth factor-betas (TGF-β) were the first negative regulators to have their gene cloned and sequenced and the expression of their mRNA detected in a mammalian cell.[1] They comprise a large number of structurally related polypeptide growth factors and play a prominent role in the development, homeostasis and repair of virtually all tissues in organisms.[2] TGF-β plays a relevant regulatory role in the homeostasis of early hematopoietic proliferation/differentiation. TGF-β_1 and TGF-β_2 are effective inhibitors of hematopoiesis.[3] They can selectively control the activity of different molecular regulators of normal and leukemic hematopoiesis.[4] The production of colony stimulating activity by bone marrow stromal cells and the transcription of granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA by activated T cells are inhibited by TGF-β.[5]

However, TGF-β has the ability to both stimulate and

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inhibit the proliferation, differentiation and other functions of various cells depending on the type of target cells on the context of other growth factors present. For example in human thyroid follicular cells (TFC) TGF-β stimulates the expression of both monocyte chemoattractant protein-1 (MCP-1) and GM-CSF expression, but suppresses macrophage colony stimulating factor (M-CSF) expression.[6] These results are generally important in understanding the ways in which cytokine regulation differs between different cell types. Varieties of non-hematopoietic malignant tumors have been demonstrated to secrete hemopoietic growth factors. Bladder cancer cells have been shown to secrete a variety of biological factors with no direct relation to urothelial cell origin including GM-CSF and various cytokines. The bladder carcinoma cell line, HTB 5637, produces colony stimulating factors for myeloid colony (Colony Forming Unit-Granulocyte Macrophage CFU-GM) growth from normal and malignant marrow. mRNA phenotyping by RT-PCR reveals expression of mRNA for IL-1α, IL-1β, IL-6,G-CSF, M-CSF, and GM-CSF in HTB 5637 cell line.[7] In the present study we aimed to examine the effect of TGF-β on the production of GM-CSF in human bladder tumor cell line HTB 5637.

Our data show that treatment of HTB 5637 cells with TGF-β_2 increases functional GM-CSF production on a dose-dependent basis. This suggests that TGF-β_2 is a positive regulator of GM-CSF gene expression in HTB 5637-cell line.

MATERIAL AND METHODS

Reagents

Recombinant human GM-CSF and cell culture media were purchased from Gibco BRL. Recombinant human TGF-β_2, DIG-High prime DNA labeling and detection starter kit II and restriction enzymes, Eco R1 and Bam H1 were obtained from Boehringer Mannheim. HTB 5637 cells (ATCC HTB 9) and also plasmids were obtained from American Type Culture Collection. Fetal calf serum was from Sigma and Ficoll-Hypaque was purchased from Flow Laboratories. Culture dishes and flasks were from Nunc and other laboratory reagents were obtained from Sigma, Merck and Fluka. hGM-CSF enzyme linked immunosorbent assay (ELISA) kit was from genzyme.

Cell culture

Monolayer cell cultures were maintained in RPMI 1640 in culture flasks or multiwell dishes containing 10% fetal calf serum. Cultures were allowed to reach 75% confluency before any experiment was carried out. Cells (1×10^5) were grown in 25-cm^2 flasks or (8×10^3) in 24-well dishes at 37°C under 7.5% CO_2. Near confluent cultures were subsequently maintained by trypsinization and subculture.

Collection of cells

Media from near confluent flasks were removed; cells were washed with fresh culture media, and incubated in media with or without 5ng/mL of recombinant human TGF-β_2. Then media from cultures was removed, centrifuged, and filtered through 0.2 μm filter paper and saved for ELISA. Total or cytoplasmic RNA was extracted from adherent layer cells.[8,9]

ELISA

Collected supernatants were diluted to appropriate (1:1 to 1:100) concentrations, and then the amount of GM-CSF was determined using ELISA kit for hGM-CSF, according to the instructions provided by the manufacturer.

Probe preparation

The P91023 and PHF (A1 plasmids containing hGM-CSF cDNA[10] and hγ-Actin full-length cDNA insert respectively, were amplified in their hosts and purified recruiting standard protocols.[11] After restriction digestion (P91023 with EcoR1) a 0.8-kb human GM-CSF cDNA insert and (PHFγA1 with BamH1) 2.1-kb hγ-Actin full-length cDNA were purified by agarose gel electrophoresis and phenol: chloroform extraction. Extracted inserts (0.5-1μg) were labeled with Boehringer Mannheim DIG labeling and detection kit, according to the instructions provided by the company.

RNA isolation and slot blot analysis

RNA was extracted from near confluent cells, which were treated with 5ng/ml of TGF-β_2, according to Chomczynski and Sacchi.[8] The quality of the RNA preparations was checked by 1.5% non-denaturing agarose gel electrophoresis for the presence of 2:1 ratio of the 28S to 18S bands, indicating the intactness of the RNA preparations. Total RNA (10μg) was blotted on positively charged nylon membrane using a Schleicher and Schuell slot blot apparatus. The membrane was prehybridized for 3 hours and then incubated in hybridization buffers containing 5-25 ng/mL labeled probe for 18 hours. Following hybridization, post hybridization stringent washes and detection, membrane was transferred to development folder and incubated with chemiluminescent substrate. Then folders were exposed to X-ray films being developed after 20 minutes.

Northern blot analysis

Cytoplasmic RNA was isolated from control and treated cultures, exposed to TGF-β2 for 24 hours, according to the protocol of Clemens.[9] The procedure is based on solubilization of the plasma membrane while maintaining nuclear integrity. Cellular lysis is accomplished as quickly as possible, on ice, in the presence of RNase inhibitors, the inclusion of which must be maintained until phenol:chloroform mixtures are introduced into the system to remove the protein. Spectrophotometric and electrophoretic analysis of the samples checked the amount and integrity of RNA. Cytoplasmic RNA (25μg) was size fractionated on 1% agarose/

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formaldehyde gels, then transferred to nylon membrane using capillary transfer. Subsequently, the nucleic acids were immobilized by backing 2h at 80°C. Blots were prehybridized for 3 hours and probed with DIG labeled probes (for hGM-CSF and hγ-Actin) at 68°C for 16-19 hours. Filters were washed to a stringency of 0.5x SSC, 0.1% SDS at 68°C. Following detection, membrane was transferred to development folder and incubated with chemiluminescent substrate, and then folders were 25-40 minutes exposed to Fuji X-ray films and films were developed.

Biological assay

The supernatant of control and treated cultures was collected 24 hours post stimulation. Biological activity was assayed in a microplate system. Human bone marrow was obtained from normal volunteer donors. Mononuclear cells were collected by centrifugation over Ficoll-Hypaque.[12] Cells (5×10^4) were plated in each well of a 96-well flat-bottomed microplate in the presence of 10 μL culture media (and/or 40 units of recombinant hGM-CSF, as CSF) and 100 μL enriched IMDM supplemented with 20% fetal calf serum, antibiotics, and 0.3% agar. Cultures were incubated for 9-11 days, in a fully humidified atmosphere of 7.5% CO_2 at 37°C. Colonies with more than 50 cells were scored using a Zeiss inverted microscope.

RESULTS

GM-CSF concentration time-course study

In order to determine the amount of GM-CSF produced by HTB 5637-cell line, cells were cultured as described in the *Methods* section in 24 well plates. Everyday at least three wells were removed from culture. Time course study with specific ELISA detected high amounts of GM-CSF in the supernatant of HTB 5637-cells culture, at the log phase of their growth curve. Results showed that increase in GM-CSF concentration was parallel to increase of cell number (Fig. 1).

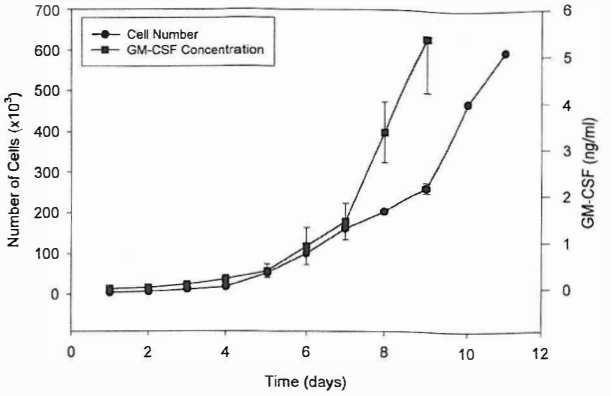


Fig. 1. Growth curve of the HTB 5637-cells and GM-CSF content of culture media. Data was presented as the mean value ±S.E. derived from three independent experiments.

The influence of TGF-β_2 on GM-CSF production: dose-response study

After removal of media, cells were washed once with complete media and were incubated in media supplemented with TGF-β_2 (0-5ng/mL). Media was removed after 48 hours and GM-CSF concentration was determined in duplicate by ELISA. Results showed a significant increase of GM-CSF in the treated samples (Fig. 2). The most considerable activity was seen at the concentration of 5 ng/mL, with higher doses of TGF-β_2 (up to 10 ng/mL) showing the same stimulating effect; this guided us to employ the 5 ng/mL dose in other experiments.

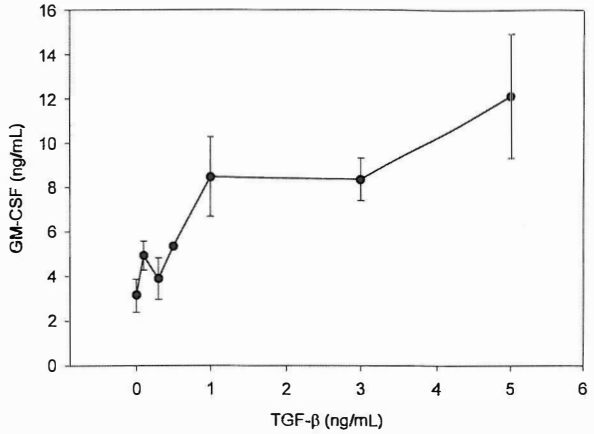


Fig. 2. TGF-β_2 stimulates GM-CSF production: a dose dependent study. Cell cultures with 70-80% confluency were washed with complete media and incubated for 48 hrs with different doses of TGF-β_2. Then the supernatant of cultures was removed and assayed for GM-CSF concentration. Data was presented as the mean value ±S.E. derived from two independent experiments.

The influence of TGF-β_2 on viable cell count and GM-CSF production: time study

Cultures were treated with or without 5 ng/mL of TGF-(2 and at each time interval, culture media was removed and saved for ELISA test. The viable cell number of each sample was also determined. Our data showed that although the number of cells in treated and control groups was the same (Fig. 3), the amount of GM-CSF production in treated samples began increasing significantly from 10 hours post stimulation, reaching the highest stimulation level at 48 hours (Fig.

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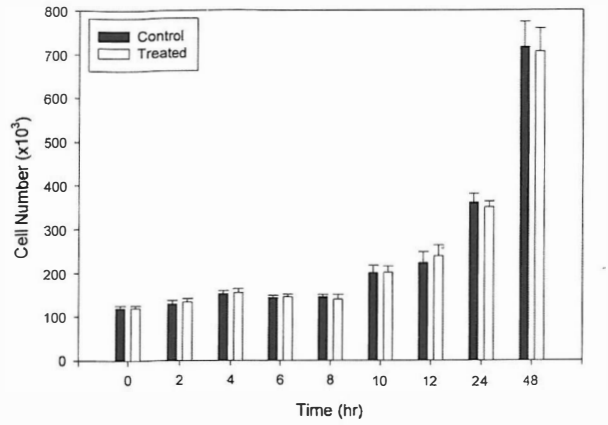


Fig. 3. Number of viable HTB 5637 cells in TGF-β_2 stimulated and unstimulated cultures. Data was presented as the mean value ±S.E. derived from three independent experiments.

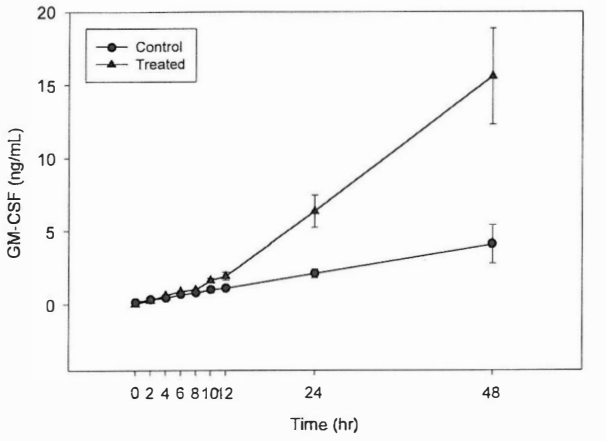


Fig. 4. The influence of TGF-β_2 on GM-CSF production: A time-course study. Cultures were incubated at the presence or absence of 5ng/mL TGF-β_2. At the indicated times the supernatant of the cultures was removed and saved for ELISA. Data are shown as the mean value ±S.E. derived from three independent experiments.

4).

Biological activity of GM-CSF

Twenty-four hours exposure to TGF-β_2 increased the number of CFU-GM colonies which were harvested from the supernatant of cultures. This increase (95%) was lower than that expected from ELISA results (200% increase for 24-hrs exposure). So in another experiment, recombinant human GM-CSF (400 unit/mL of assay media) was added to control and treated cells culture media. Interestingly the number of CFU-GM colonies for control culture media increased and reached that harvested from treated cells cul-

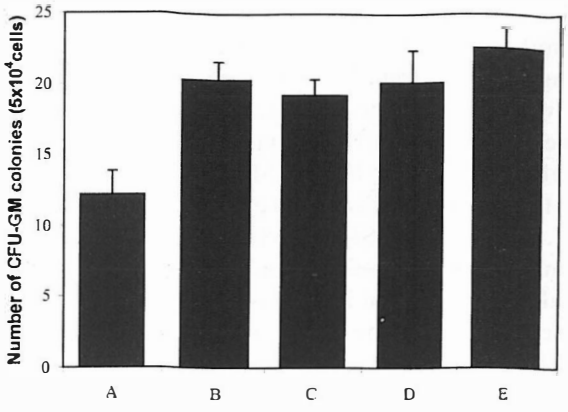


Fig. 5. Effects of TGF-β_2 on the CSA activity of the culture media. The number of CFU-GM colonies which formed in the presence of 10% control culture media (A), or 10% treated cells culture media (24-hrs exposure to TGF-β2) (B). The number of colonies formed in the presence of A + 400 u/mL rhGM-CSF (C) and B + 400 u/mL of rhGM-CSF (D). CFU-GM colonies formed in the presence of 400u/mL rhGM-CSF (E). Data was presented as the mean value ±S.E derived from two independent experiments.

ture media, whereas the number of CFU-GM colonies harvested from treated cells culture media (in the presence and/or absence of rhGM-CSF) was the same (Fig 5).

TGF-β_2 stimulates expression of GM-CSF mRNA in HTB 5637- cell line

Total RNA was extracted from treated and controls samples and Slot-Blot experiment was performed. Results clearly showed an increase in GM-CSF mRNA content from treated cells. This effect was not seen for hγ-Actin (Fig. 6). Quantitative analysis of slots was performed using a densitometer (Table I). According to slot blotting data, 24-hrs exposure to TGF-β_2 increased GM-CSF transcripts by 50%. We analyzed the effect of TGF-β_2 on the expression of GM-CSF mRNA by performing Northern blot analysis of cytoplasmic RNA extracted from HTB 5637 cells which had been treated with 5ng/mL of TGF-β_2 for 24 hrs. Results clearly showed an increase in the GM-CSF mRNA content from treated cells (Fig 8). There appeared to be a slight change in actin mRNA levels from the analysis of the Northern blot. However, this discrepancy may be due to differences in the amount of cytoplasmic RNA loaded on this particulate gel (Fig. 7). Experiments have confirmed that TGF-β_2 does not alter actin mRNA levels as expected.

DISCUSSION

TGF-β, like many other peptide growth factors, is mul-

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Table I. The ratio of treated: control mRNA content for γ-Actin or GM-CSF slot blot analysis of total RNA.

Hours post stimulation	GM-CSF mRNA Treated/Control	γ-Actin mRNA Treated/Control
10	1.1	1
12	1.2	1
24	1.5	0.9
48	1.5	1

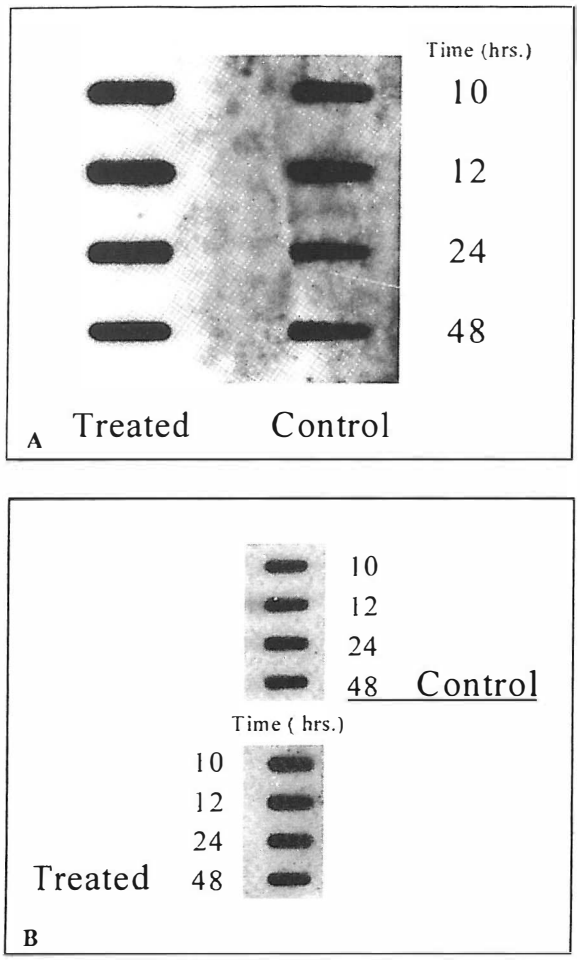


Fig. 6. Slot blot analysis of total RNA. a time-course study: 10μg of total RNA, from control and treated cultures, was loaded on slots. The membrane was hybridized with specific DIG labeled probe.A; GM-CSF mRNA content, B; γ-Actin mRNA content.

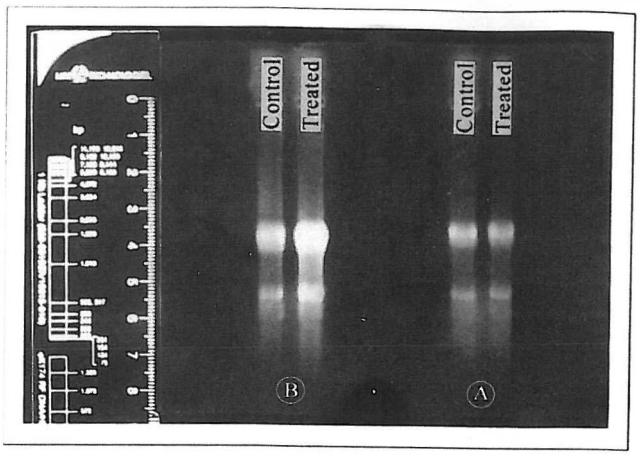


Fig. 7. Cultures were exposed to TGF-β_2 for 24-hrs, cytoplasmic RNA was extracted and 25 μg of cytoplasmic RNA was loaded on each lane of the gel. (A) The gel was duplicated. Ethidium bromide stained gel, which was used for Northern analysis of hGM-CSF mRNA. (B) Ethidium bromide stained gel, which was used for Northern analysis of γ-Actin mRNA; on this particulate gel, a higher level of cytoplasmic RNA was loaded from the treated sample.

tifunctional and many of its most important activities have little to do with the transformation system in which it was first discovered. Much of the current interest in TGF-β research reflects its importance as a mediator of inflammation, repair and angiogenesis, as well as its importance as a negative regulator for many epithelial cells and for both T and B-lymphocytes.[13,14] Because essentially all cells have receptors for TGF-β [15,16] TGF-β has the potential to regulate physiological function in almost all tissues of the body.

In various hemopoietic systems TGF-β has been shown to play an inhibitory role on the production of cytokines.[17,18] It suppresses the production of colony-stimulating activity by bone marrow stromal cells and the synthesis of GM-CSF by activated T cells, apparently at a pretranslational level.[5] TGF-β may be an active suppressor factor regulating hemopoiesis. It is supported by the reported inhibition of GM-CSF mRNA transcription in a T cell line by the TGF-β.[19] It is also possible that TGF-β may induce the production of other suppressive factors.

However these inhibitory functions seem to be tissue dependent and there are several reports on the stimulating activity of TGF-β on CSF production by tissues (or cells) other than hemopoietic types. For example in human thyroid follicular cells (TFC) and human retinal pigment epi-

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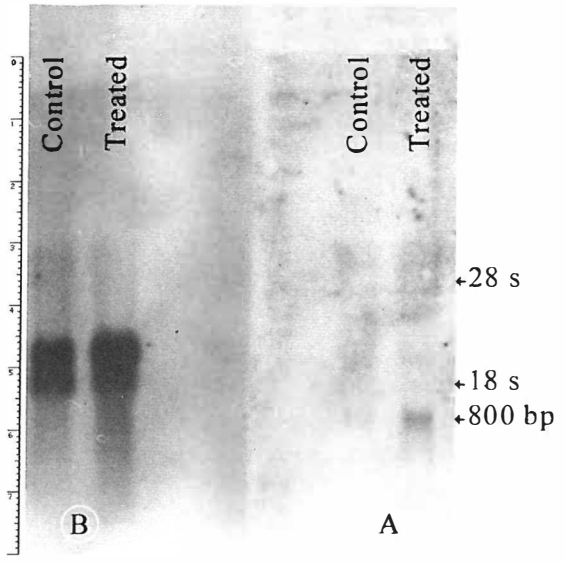


Fig. 8. TGF-β_2 stimulates GM-CSF transcripts in the HTB 5637-cell. Northern blot analysis of cytoplasmic RNA. After blotting the membrane was hybridized with GM-CSF cDNA probe (A). After blotting, the membrane was hybridized with γ-actin cDNA probe (B). An increase in the level of γ-actin mRNA in the treated sample was related to overloading.

thelial cells TGF-β_2 stimulates the expression of GM-CSF.[6,20]

Here we report an instance in which TGF-β stimulates GM-CSF production in a non-hemopoietic system. We recruited human bladder tumor cell line HTB 5637 as a reliable and potent source for GM-CSF production. RNA phenotyping for colony stimulating factors by reverse transcription and polymerase chain reaction reveals the expression of mRNA for GM-CSF along with a range of other cytokines, in RNA preparation of these cells.[7]

In contrast to other reports showing that TGF-β inhibits the proliferation of normal and certain tumor derived epithelial cell lines,[21] we didn't observe any antiproliferative effect of TGF-β_2 on the HTB 5637-cell line.

Quite interestingly we confronted an increase in the number of CFU-GM colonies which was derived from a biological assay of the treated (24 hrs) cells media. However this increase (95%) was lower than that expected from ELISA (200%). Since TGF-β_2 does not have a remarkable effect on hemopoietic progenitor cells[22] it seems that some inhibitory factor(s) may produce an increase in response to TGF-β_2 treatment.

The mechanism of this inhibition requires further study. Analysis of RNA stability has shown that the GM-CSF mRNA content of HTB 5637-cell line is very stable with a half-life of four hours.[23] The stabilization of GM-CSF mRNA in these cells is translational dependent.[24]

Both Slot blot and Northern analysis showed an increase in GM-CSF mRNA in treated cells. Concluding from these results we would like to suggest a stimulating role for TGF-β in increasing GM-CSF production through changes adapted at the mRNA level. These observations may introduce TGF-β as a positive regulator for GM-CSF gene expression in HTB 5637-cell line.

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