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In Vitro Bioassay for Transforming Growth Factor-β Using XTT Method

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Research in the cytokine field has grown exponentially in recent years, and the validity of such studies relies heavily on the appropriate measurement of levels of cytokines in various biological samples. Transforming growth factor (TGF)-β, a hormonally active polypeptide found in normal and transformed tissue, is a potent regulator of cell growth and differentiation. The most widely used bioassay for TGF-β is the inhibition of the proliferation of mink lung epithelial cells. Though detection of [^{3}H]thymidine incorporation is more sensitive than the MTT assay, it presents some disadvantages due to the safety and disposal problems associated with radioisotopes. In this study, we attempted to ascertain the experimental conditions which could be used for measuring the *in vitro* biological activity of TGF-β in a safer and more sensitive way compared with the currently available methods. We compared the commonly used method, the MTT assay, to the XTT assay using different parameters including cell number, incubation time and the wave length used for detecting the product. We examined the anti-proliferative activities of TGF-β in three different cell lines: Mv-1-Lu mink lung epithelial cells, MCF10A human breast epithelial cells and H-ras-transformed MCF10A cells. Herein, we present an experimental protocol which provides the most sensitive method of quantifying the biological activity of TGF-β, with a detection limit of as low as 10 pg/ml: Mv-1-Lu or H-ras MCF10A cells (1×10^5/well) were incubated with TGF-β at 37°C in a humidified CO_2 incubator for 24 hr followed by XTT treatment and determination of absorbance at 450 or 490 nm. Our results may contribute to the establishment of an *in vitro* bioassay system, which could be used for the satisfactory quantitation of TGF-β.

Key words: TGF-β, Mv-1-Lu, H-ras MCF10A, MTT, XTT

INTRODUCTION

The advent of recombinant DNA technology and its application in the pharmaceutical industry has led to the rapid growth of biotechnology companies and a number of biological medicinal products are already available or are under development (Cuthbert *et al.*, 1988). Many different proteins including cytokines (Gillio *et al.*, 1993) have been produced using this technology. Research in the cytokine field has grown exponentially in recent years, particularly since it was revealed that cytokines play a role in the pathogenesis of many clinical disorders as well as showing therapeutic potential for the treatment of a wide range of diseases (Mire-Sluis *et al.*, 1995). The validity of such research relies heavily on the appropriate measurement of levels of cytokines in various biological samples.

Transforming growth factor (TGF)-β is a 25 kDa homodimer producing a wide range of biological effects depending on the target cell type and the experimental conditions (Roberts and Sporn, 1990). Several different subtypes of TGF-β have been described. In mammals, there are three major subtypes designated TGF-β1, TGF-β2 and TGF-β3 (Barnard *et al.*, 1990). Despite the structural differences between these TGF-β subtypes, they have been found to exhibit similar behavior in most assays (Graycar *et al.*, 1989). For some cells TGF-βs stimulate proliferation, but in most cases they are potent inhibitors of cell growth and proliferation (Meager, 1991). TGF-β has been implicated as being a mediator in a range of pathological disorders involving cancer, inflammation and immunosuppression (Border *et al.*, 1990; Fava *et al.*, 1989; Wahl, 1992). Several biological and immunological assays are available to test for the presence of TGF-β.

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Although immunoassays are useful indicators of the presence of this cytokine, they reveal nothing about the biological activity of the molecule and this is particularly important for a cytokine such as TGF-β since it is secreted in an inactive from that has to be subsequently activated by proteolytic cleavage (Randall *et al.*, 1993).

TGF-β is capable of inducing the proliferation of certain murine cell lines (Moses *et al.*, 1981; Rizzino, 1987) and the proliferation assays currently in use rely on the capacity of TGF-β to induce anchorage-independent proliferation of normal or untransformed fibroblasts in the presence of other growth factors in soft agar to form colonies (Roberts *et al.*, 1982; Julien *et al.*, 1989; Kothapalli *et al.*, 1997). Since this type of assay requires the use of laborious qualitative procedures prone to error in order to enumerate the colonies, it is no longer the method of choice amongst the available TGF-β bioassay procedures.

A widely used bioassay for TGF-β is the inhibition of proliferation of the Mv-1-Lu mink lung epithelial cell line which is sensitive to approximately 50 pg/ml of TGF-β by means of [^{3}H]thymidine incorporation or the MTT staining technique (Meager, 1991; Amatayakul *et al.*, 1994). Another sensitive assay for TGF-β, using a radioisotope, was developed based on the ability of TGF-β to inhibit the interleukin-5-induced proliferation of a human erythroleukemic cell line, TF-1 (Randall *et al.*, 1993). Though the detection of [^{3}H]thymidine incorporation is more sensitive than the MTT assay, it also has some disadvantages, in that the safety and disposal problems associated with radioisotopes have to be overcome. Recently, a new colorimetric assay method, XTT assay, has been developed (Meshulam *et al.*, 1995; Hawser *et al.*, 1998).

The purpose of this study was to ascertain the experimental conditions which could be used for measuring the *in vitro* biological activity of TGF-β in a safer and more sensitive way, as compared to the currently available methods. Herein, we present an experimental protocol which is more sensitive to the biological activity of TGF-β than the existing methods, with a detection limit of as low as 10 pg/ml, and which may contribute to the establishment of an *in vitro* bioassay system which can be used for the quantitation of TGF-β.

MATERIALS AND METHODS

Cell lines and culture condition

Stock cultures of Mv-1-Lu mink lung epithelial cells (CCL-64, from ATCC) were grown in minimal essential media supplemented with 5% fetal bovine serum and non-essential amino acids. MCF10A is a spontaneously immortalized "normal" breast epithelial cell line (Soule *et al.*, 1990). Establishment of the H-ras MCF10A cells was previously described (Moon *et al.*, 2000). MCF10A and H-ras MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml EGF, 0.1 μg/ml cholera enterotoxin, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 0.5 μg/ml fungizone. The cells were cultured in a humidified 5% CO_2 incubator at 37°C.

Materials

Human TGF-β1 interim standard (89/514) was obtained from the National Institute for Biological Standards and Control (NIBSC, UK) and resuspended in distilled water to make a 200 μM stock solution. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) and PMS (Phenazine methosulfate) were purchased from Sigma Chemical Co.

MTT assay

Cells were harvested, counted, and inoculated at the appropriate concentrations (100 μl) into 96-well plates using a multichannel pipet. After 24 hr, TGF-β was applied to the culture wells, and the cultures were incubated for 24 hr and 48 hr at 37°C, under the atmosphere of 5% CO_2. MTT solution with a concentration of 5 mg/ml was added to the media and the cells were further incubated for 4 hr. After 100 μl of supernatant was replaced with 100 μl of DMSO, the absorbance of each well was determined at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA). The percentage of cell survival was defined as the relative absorbance of the treated versus the untreated cells.

XTT assay

The XTT assay methodology was essentially the same as that involving the use of the MTT reagent with the following modifications (Scudiero *et al.*, 1988). Twenty-five μl of 5 mM PMS stock solution were added per 5 ml of XTT stock solution (1mg/ml) to make 0.025 mM of PMS-XTT solution. Fifty μl of this mixture were added each well after 24 hr or 48 hr of cell incubation. Subsequently, after 4 hr of incubation at 37°C, the plates were mixed and the absorbance was measured. The viability of the cells was estimated on the basis of the quantity of formazan formed, which was measured spectrophotometrically (Logu *et al.*, 2001).

RESULTS

The Mv-1-Lu mink lung epithelial cell line whose proliferation is strongly inhibited by TGF-β has been reported to be a suitable cell line for the anti-proliferation type of TGF-β bioassay. In order to identify sensitive cell lines of human origin which could be used for measuring the anti-

proliferative activity of human TGF-β, we examined the effect of TGF-β on proliferation of MCF10A human breast epithelia cells and their transformed counterpart, H-ras MCF10A cells. Although the MTT assay has been commonly used for the TGF-β bioassay without using radioisotopes, it presents some drawbacks in that it requires the centrifugation of the cells and the removal of the medium, due to the formation of precipitates. We used the XTT assay instead, which has the advantage of yielding a water-soluble formazan product after reduction, and thus can be easily quantified without performing additional steps such as the centrifugation of the cells and the removal of the medium (Logu *et al.*, 2001).

The growth inhibition assay was carried out in 96-well microtiter plates. Figs. 1, 2, and 3 show the growth curves which were obtained when Mv-1-Lu, MCF10A and H-ras MCF10A cells were seeded at low cell density (1×10^3, 5×10^3 and 1×10^4 cells/well, respectively) in growth medium containing serial dilutions of TGF-β standard. The assays were incubated at 37°C in a humidified CO_2 incubator for 48 hr followed by XTT treatment and determination of the absorbance at different wavelengths. Though a slight reduction of cell viability was observed in the Mv-1-Lu cells (1×10^3 cells/well) when the absorbance was determined at 490 nm (Fig. 1), the sensitivity obtained was not satisfactory, with only a 25% reduction in proliferation being detected by 50 pg/ml TGF-β. No anti-proliferative effect of TGF-β was detected in any of the cell lines tested when the cell numbers were 5×10^3 cells/well (Fig. 2) or 1×10^4 cells/well (Fig. 3).

Reducing the incubation time to 24 hr did not allow the dose-dependent measurement of anti-proliferative activity of TGF-β, when the assays were performed with 1×10^4 cells/well (Fig. 4). Fig. 5 shows the results of the experimental protocol in which a higher density of cells (1×10^5 cells/well) were incubated with various concentrations of

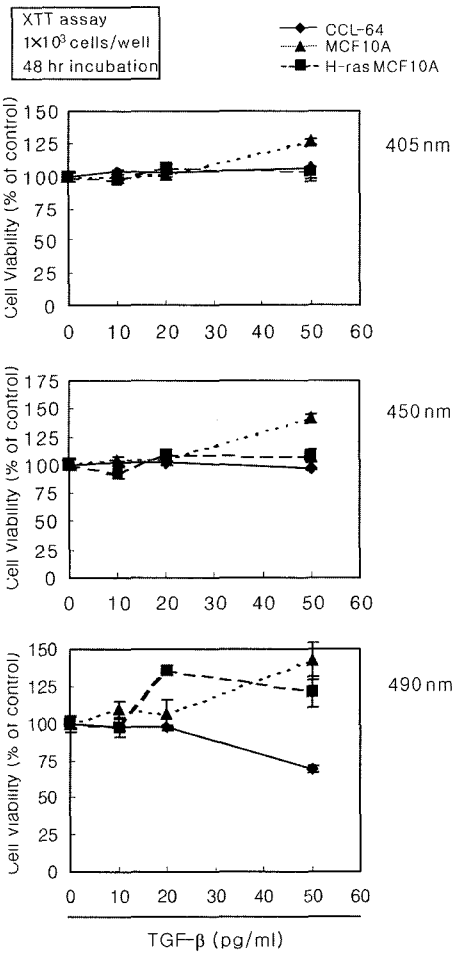

Fig. 1. Effect of TGF-β on cell proliferation when 1×10^3 cells were treated with TGF-β for 48 hr. XTT assay was performed and the growth inhibition was determined by relative absorbance at 405, 450 or 490 nm normalized to the control cells. The results presented are means± S.E. of triplicates.

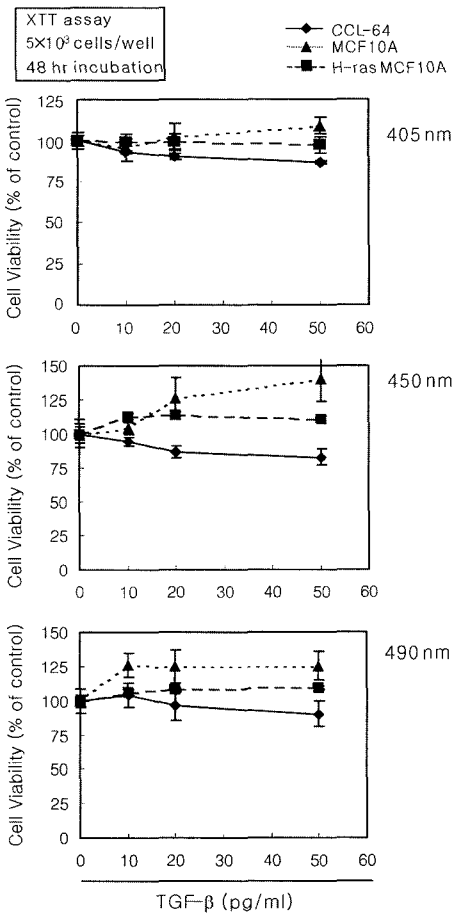

Fig. 2. Effect of TGF-β on cell proliferation when 5×10^3 cells were treated with TGF-β for 48 hr. XTT assay was performed and the growth inhibition was determined by relative absorbance at 405, 450 or 490 nm normalized to the control cells. The results presented are means± S.E. of triplicates.

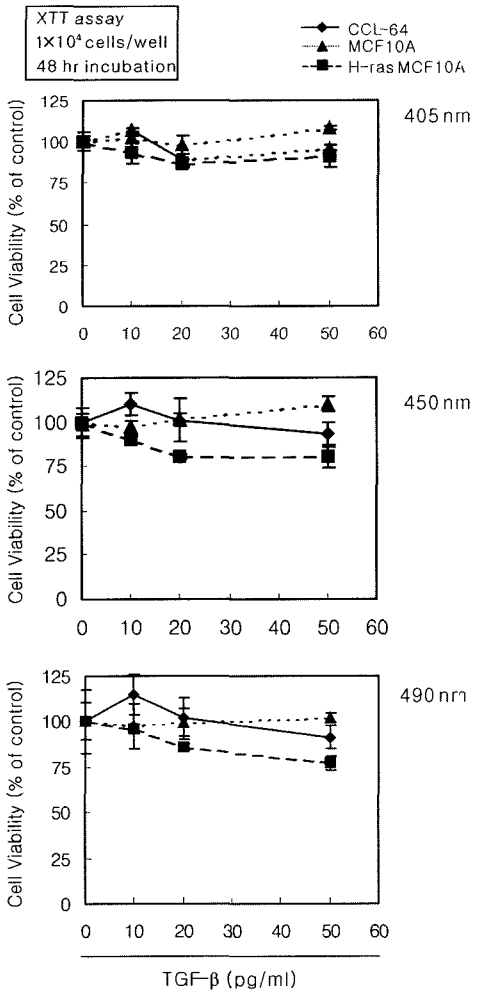

Fig. 3. Effect of TGF-β on cell proliferation when 1×10^4 cells were treated with TGF-β for 48 hr. XTT assay was performed and the growth inhibition was determined by relative absorbance at 405, 450 or 490 nm normalized to the control cells. The results presented are means± S.E. of triplicates.

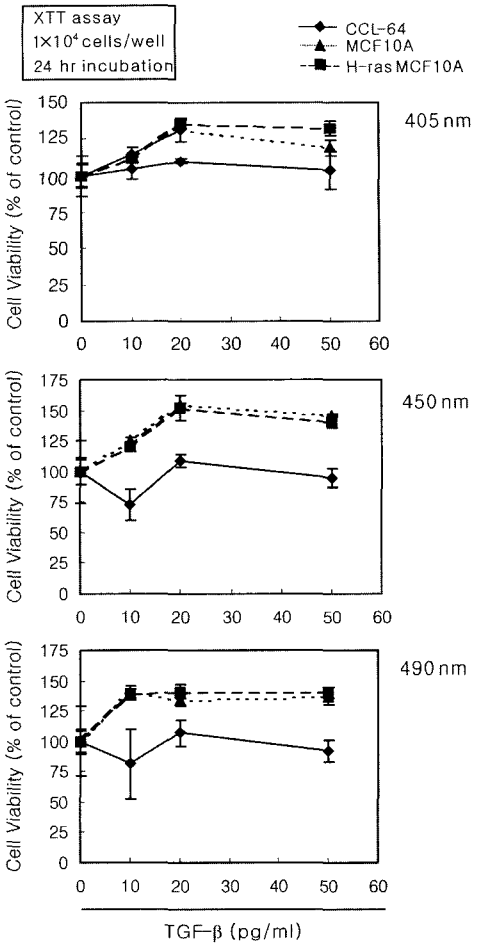

Fig. 4. Effect of TGF-β on cell proliferation when 1×10^4 cells were treated with TGF-β for 24 hr. XTT assay was performed and the growth inhibition was determined by relative absorbance at 405, 450 or 490 nm normalized to the control cells. The results presented are means± S.E. of triplicates.

TGF-β for 24 hr. When the soluble products were detected at 405 nm, an inhibitory effect of TGF-β on cell proliferation was observed in the Mv-1-Lu cells, but not in the MCF10A or H-ras MCF10A cells. Determination of absorbance at 450 and 490 nm, however, enabled the dose-dependent detection of the anti-proliferative activity of TGF-β not only in the Mv-1-Lu cells but also in the H-ras MCF10A cells. MCF10A cells were not responsive to treatment with TGF-β at any of the concentrations tested in this study (up to 50 pg/ml). H-ras MCF10A cells were more sensitive to the anti-proliferative activity of TGF-β than the Mv-1-Lu cells especially when a low dose of TGF-β was used. At 450 nm, treatment with 10 pg/ml of TGF-β caused ~60% inhibition of H-ras MCF10A cell proliferation whereas the same treatment resulted in only 10% inhibition of Mv-1-Lu cell proliferation. When the cells were treated with a higher dose of TGF-β, however, the CCL-64 cells were more sensitive than the H-ras MCF10A cells. Similar results, concerning the anti-proliferative effect of TGF-β, were observed when the absorbance was measured at 490 nm. No dose-dependent measurement of TGF-β activity was found in any of the cell lines, when MTT was used in place of XTT (Fig. 6), suggesting that the XTT assay constituted a more sensitive method than the MTT assay in our experimental system.

In conclusion, we were able to find an experimental protocol, which provides the most sensitive method of quantifying the biological activity of TGF-β: 1×10^5 cells (Mv-1-Lu or H-ras MCF10A cells) were plated on a 96-well plate and the media was changed to serum free media (phenol red-free) containing various concentrations of TGF-β in pg/ml. Following 24 hr incubation, XTT was treated for 4 hr at 37°C, 5% CO_2 and the absorbance was determined at 450 or 490 nm.

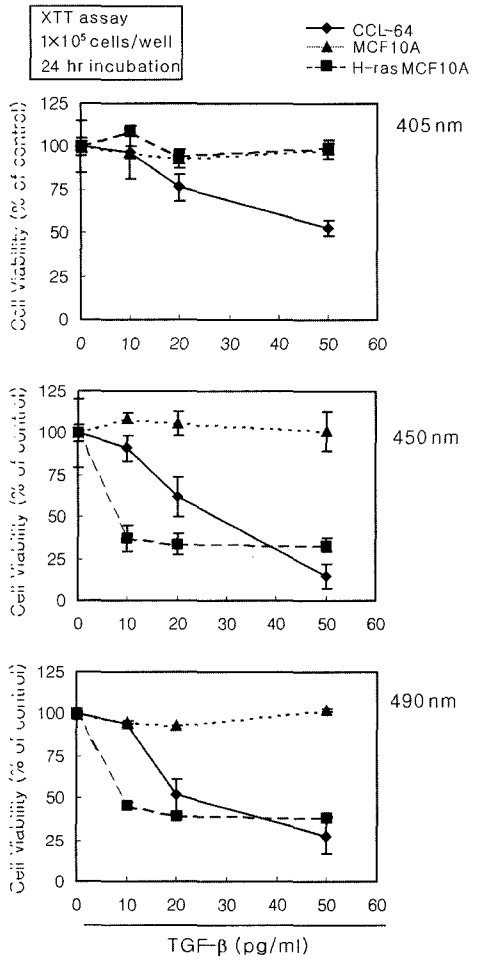

Fig. 5. Effect of TGF-β on cell proliferation when 1×10^5 cells were treated with TGF-β for 24 hr. XTT assay was performed and the growth inhibition was determined by relative absorbance at 405, 450 or 490 nm normalized to the control cells. The results presented are means± S.E. of triplicates.

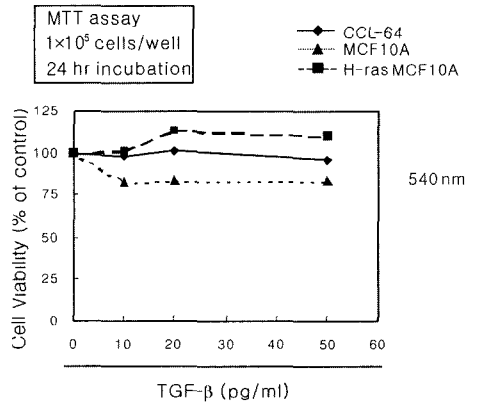

Fig. 6 Effect of TGF-β on cell proliferation when 1×10^5 cells were treated with TGF-β for 24 hr. MTT assay was performed and the growth inhibition was determined by relative absorbance at 540 nm normalized to the control cells. The results presented are means ± S.E. of triplicates.

DISCUSSION

Due to the wide spectrum of biological activities exhibited by TGF-β, a wide choice of parameters is available for its determination. TGF-β bioassays in which cell proliferation is stimulated or inhibited are commonly used. The most widely used bioassays for TGF-β measure the *in vitro* anti-proliferative activity of TGF-β. In this study, we attempted to find an experimental protocol which would provide the most sensitive method of quantifying the biological activity of TGF-β. We used human TGF-β1 interim standard for our assays. Since different TGF-β subtypes have been found to behave similarly in most assays (Graycar *et al.*, 1989), it can be assumed that the results of this study also apply to assays used for other TGF-β subtypes. The MTT and XTT colorimetric assays are rapid, reliable, and affordable and do not require the use of a radioisotope. It has been shown that the results obtained with the XTT assay are comparable to those obtained with the MTT assay (Logu *et al.*, 2001). Our data show that the XTT assay is more sensitive for the measurement of the *in vitro* anti-proliferative activity of TGF-β than the commonly used MTT assay when using the Mv-1-Lu and H-ras MCF10A cell lines. Furthermore, it must be emphasized that the reduction of XTT yields a water-soluble formazan product, thus eliminating the need for additional steps.

We examined the possible inhibitory effect of human TGF-β on the growth of a cell line of human origin, MCF10A. Proliferation of MCF10A cells was not affected by TGF-β at doses of 0-50 pg/ml. Transfection of MCF10A cells with viral H-ras was demonstrated to induce a transformed phenotype, as evidenced by the induction of anchorage-independent growth and invasive phenotype (Moon *et al.*, 2000). In this study, we present the H-ras MCF10A cell line as a sensitive cell line for detecting the anti-proliferative activity of TGF-β, especially for samples containing <10 pg/ml of TGF-β. Despite TGF-βs having a high degree of conservation of its primary structure across different animal species and a high degree of cross-species activity (Meager, 1991), a cell line of human origin may be advantageous for the bioassay of human TGF-β. The optimal conditions for the assay using the H-ras MCF10A cell line was determined as being a cell number of 1×10^5/well, incubation time of 24 hr, absorbance at 450 or 490 nm (Fig. 5) and the use of XTT rather than MTT as an assay method (Fig. 6). Much remains to be done to ensure the maintenance, stability and specificity of the H-ras MCF10A cell line when used for the TGF-β bioassay.

Interestingly, the anti-proliferative activity of TGF-β in H-ras MCF10A cells was detected neither when the soluble product was measured at 405 nm nor when the cell number was 1×10^4 cells/well. A dose-dependent inhibition of

Mv-1-Lu cell proliferation by TGF-β was detected at 450 and 490 nm, rather than at 405 nm. *In vitro* bioassays are greatly influenced by such experimental parameters, and hence it is of importance to determine the optimal conditions for measuring the biological activities of cytokines such as TGF-β in each cell line.

In addition to the anti-proliferation assay described above, alternative bioassays could be designed which depend on the TGF-β-induced alterations in the cellular matrix formation or in the expression/secretion of cellular proteins. TGF-β has been reported to have a signaling role in the invasiveness and metastasis of carcinoma (Oft *et al.*, 1998). TGF-β was shown to promote invasiveness after cellular transformation with activated ras in intestinal epithelial cells (Fujimoto *et al.*, 2001). Recent evidence shows that TGF-β induces the expression of matrix metalloproteinases (MMPs), which degrade components of the extracellular matrix and are thus involved in cell invasiveness (Wick *et al.*, 2001; Chang *et al.*, 2001; Eichler *et al.*, 2002). We observed that TGF-β promotes invasiveness and MMP expression in certain cell lines in a dose-dependent manner, suggesting the possibility of developing a new bioassay for measuring the biological activity of TGF-β (data not shown). Further studies to determine the most appropriate conditions for this type of assays and to identify cell lines which are both sensitive and specific are currently under investigation.

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