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Mitogenic Action of TGF-β and Insulin in L-929 Cell Line in Serum-Free Medium

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In serum-free medium, TGF-β, in a wide range of concentrations, stimulated DNA synthesis. A similar effect was achieved with insulin even after relatively short times. When TGF-β and insulin were present simultaneously, the mitogenic effect was stronger than the effect achieved by either one separately, but without synergism. The PDGF, which is not mitogenic by itself in this cell line, did not increase the response to TGF-β. In the presence of fetal bovine serum TGF-β and insulin DNA synthesis was not stimulated. Two of the most important mitogenic growth factors for L-cells present in serum could be insulin and TGF-β. Adenosine did not modify the mitogenic response to TGF-β and insulin. However, in the presence of adenosine PDGF stimulated the growth of L-929. The results suggest that TGF-β does not stimulate the growth of L-929 via an autocrine production of PDGF-related peptides in a serum-free model. TGF-β blocked the inhibitory response to estradiol at high concentrations, but it did not affect the inhibitory response due to glucocorticoids. Insulin and TGF-β caused an enhancement of β-NGF and c-myc RNA expression. This effect appears much earlier with insulin. This difference suggests that mRNA accumulation provoked by TGF-β is mediated by other factors. Fetal bovine serum had little effect on the expression of those two mRNAs.

Key words: TGF-β, Insulin, PDGF, β-NGF, Mitogenic action.

Transforming growth factor, TGF-β, has been described as a multifunctional modulator, positive or negative, which affects cell proliferation (1, 24). Studies carried out in different laboratories have shown that this factor stimulates the proliferation of cells originated in the mesenchima (41, 43), and inhibits that of epithelial or endothelial cells (15, 45). TGF-β is also known to inhibit the growth

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of T and B lymphocytes as well as that of several tumour cell lines (17, 24, 36).

It has been observed that in cultures of AKR-2B using a serum-free medium a prolongation of phase G1 takes place when TGF-β is present (41). LEOF *et al.* (19) have shown that TGF-β induces the expression of the oncogene c-sis leading to an increased liberation of PDGF-like proteins (platelet-derived growth factor) to the growth medium. TGF-β provokes also the PDGF-A gene expression in human fibroblasts (31) as well as in smooth muscle cells (20). SOMA and GROTENDORST (42) have found that antibodies against PDGF block the mitogenic effect induced by this factor on primary fibroblasts from human skin. These data suggest that the mitogenic effect on fibroblasts could be mediated by an induction of PDGF-related proteins and subsequent autocrine stimulation of DNA synthesis.

TGF-β stimulates the synthesis of extracellular matrix proteins (7, 16, 32, 48). These proteins are supposed to play an important role in the mitogenic regulation of the cellular proliferation by TGF-β, either stimulating (16) or inhibiting cell growth (26).

Insulin has a direct mitogenic effect on different cell lines, and on cultures of breast cancer cells (MCF-7), where it acts as an important factor for cell proliferation at physiological concentrations (5, 28). This hormone is commonly employed in serum-free culture medium at high concentrations as a progression factor to keep cells growing without serum supplement (11).

The work here reported was aimed at the study in the cell line L-929 of the mechanism of action of TGF-β proposed by LEOF *et al.* (19) and SOMA and GROTENDORST (42) in fibroblasts. The effect of the combination of TGF-β with low concentrations of insulin was also studied (23). This cell line was obtained from adult connective tissue and turned tumoral in culture, with capacity to develop into tumours in athymic mice (13). It is known that L-929 cells synthesize TGF-β, and are responsive to it (11), and at the same time release several other factors into the medium such as β-NGF (β-nerve growth factor) (29).

Materials and Methods

TGF-β and PDGF were from British Biotechnology. Insulin was from Sigma. Medium RPMI 1640, glutamine, fetal bovine serum (FBS), trypsin (0.05 %)-EDTA (0.02 %) were purchased from GIBCO. [2-^{14}C] uridine (60 mCi/mmol), [methyl-^{3}H] thymidine (25 Ci/mmol), [γ-^{32}P]ATP (>5000 Ci/mmol) were from Amersham. T4 polynucleotide kinase was from Boehringer Mannheim. The scintillation liquid was Formula-988 from Du Pont. All other materials were from Sigma.

Cell culture. — Fibroblast-like mouse L-cells (cell line L-929) were used and maintained at 37 °C in an atmosphere of 5 % CO_2. The cells were grown in RPMI 1640, 10 % FBS and 20 mM HEPES supplemented with glutamine (2 mM) and gentamycin (50 μg/ml) and every 4 days a passage with trypsin (0.05 %)-EDTA (0.02 %) was performed.

Incorporation of precursors into DNA or RNA. — The incorporation of precursors of DNA or RNA was carried out following the method of OSBORNE *et al.* (28). Confluent cultures were suspended in trypsin-EDTA. Aliquots of this suspension containing 8.000 cells were transferred to each well into 96-well plates. After 24 hours the medium was removed and the cells were washed with RPMI 1640. Culture medium free from fetal serum was then added and the cells incubated for another period of 24 hours, at the end of which the growth factors diluted in RPMI 1640 were added. At different time intervals labelled precursors diluted in RPMI 1640 were added to give

the following concentrations: [^{3}H]-Thymidine 0.8 μCi/ml, [^{14}C]-Uridine 1.25 μCi/ml. The cells were incubated for an additional period of 2 hours, and then the medium was removed; the cells were suspended in trypsin-EDTA and collected on Skatron filters, subsequently washed with distilled water. The radioactivity incorporated was determined with a Beckman liquid scintillation counter. In some experiments FBS, to a final concentration of 10 %, was added to the medium together with the growth factors. Results were obtained from three experiments carried out in triplicate.

RNA extraction. — L-929 cells were cultured in 225 cm^2 cell culture flasks. Once confluent, cells were washed and placed in fresh serum-free medium. On the following day, the medium was again replaced with new serum-free medium and the different factors. At time intervals cells were harvested, pelletted and immediately frozen in liquid nitrogen.

Total cellular RNA isolation was performed by a guanidinium isothiocyanate solubilization and centrifugation of the extract through a cushion of 5.7 M CsCl (8).

Northern blot analysis. — Northern blots and hybridization assays were carried out using protocols already described in detail (38). Equal amounts of RNA (40 μg), measured by O. D. at 260/280, were electrophoresed through a 1 % agarose-formaldehyde gel. After electrophoresis RNA was blotted on Nytran nylon membranes (Schleicher & Schuell), dried and baked at 80 °C under vacuum. Equal amounts of RNA were loaded on each lane comparing the ethidium bromide staining of the ribosomal bands as described (19, 42).

Filters were prehybridized at 42 °C during 4 hours in 30 % formamide, 5 × Denhardt's solution (0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, and 0.1 % BSA), 5 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7), 50 mM sodium phosphate, 0.25 mg/ml heat-denatured salmon sperm DNA, and 0.25 % SDS. Hybridization was then performed at 42 °C overnight with the radiolabelled probes in 30 % formamide, 5 × SSC, 0.2 mg/ml heat-denatured salmon sperm DNA, 2 × Denhardt's solution, 20 mM sodium phosphate and 0.1 % SDS. Filters were then washed with low (2 × SSC and 0.1 % SDS) or high (0.2 % SSC and 0.1 % SDS) stringency at different temperatures depending on the affinity of the probe.

Air-dried filters were then exposed to Kodak X-Omat film at −70 °C using an intensifying screen.

Probes. — Oligonucleotide probes were synthesized on a 391 DNA Synthesizer (Applied Biosystems Inc.). The oligonucleotide probe for β-NGF was: 5'-GCA CTT GGT CTC AAA AAA GTA CTG TCT GAA TAC ACT GTT GTT AA – 3' (39). The structure of the probe for the c-myc was: 5' - TT CTC CAC AGA CAC CAC ATC AAT TTC TTC CTC ATC TTC TTG CTC TTC TT – 3' (44). Probes were labelled at the 5' end with [γ–^{32}P]ATP by T4 polynucleotide kinase using published procedures (38).

Results and Discussion

Effect of TGF-β on DNA and RNA synthesis in L-929 cells. — In any study aimed at the elucidation of the effect of mitogenic agents the use of a well defined medium is of great importance in which the presence of any other potential mitogens or modulators should be avoided (3, 4, 46). L-929 cells, when placed in RPMI 1640 medium without serum, progressively lost their mitotic capacity, although preserving complete viability, at least within the 24 hour period of the assay, as determined by trypan blue exclusion. When the cells were stimulated with insulin at a concentration of 500 ng/ml a marked mitogenic response was already observed after 12 hours (2.97 fold), reaching a maximun between 19 and

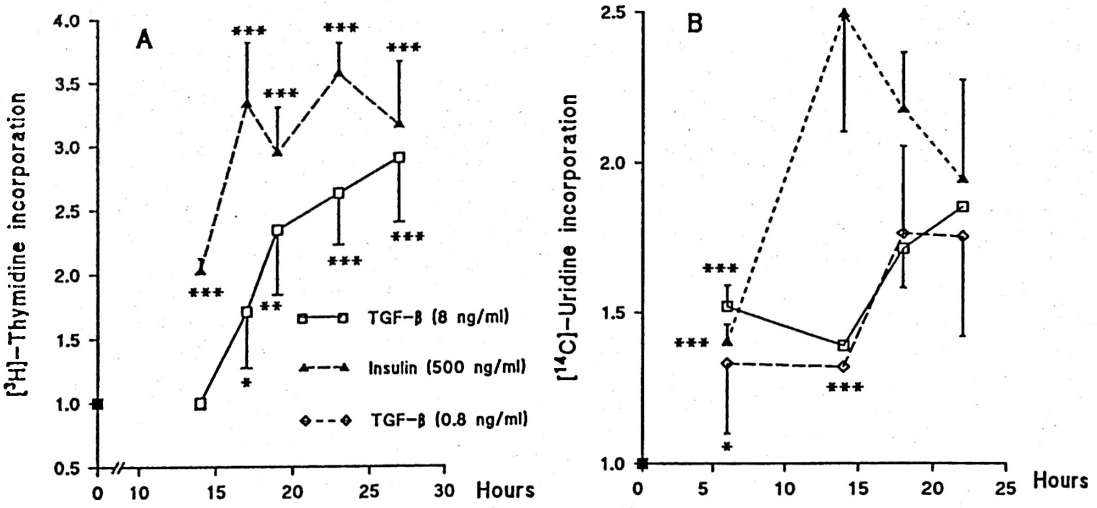

Fig. 1. *Kinetics of the effect of TGF-β and insulin on thymidine (A) and uridine (B) incorporation in serum-free medium, fold of control.*
Cells were previously maintained in serum-free medium for 24 h. Factors were added at time 0 and [^{3}H]-Thymidine or [^{14}C]-Uridine was added for a 2-h pulse before harvesting the cells at the times shown. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, as compared to no addition (Student's t test).

23 hours (fig. 1A). With TGF-β an increase in the incorporation of [^{3}H]-Thymidine was not observed until past 17 hours, and kept increasing until 30 hours later. An increase in the synthesis of RNA was observed both with insulin or TGF-β as early as 6 hours (fig. 1B). Therefore, both factors provoked a mitogenic effect on the cell line L-929, with a delay in the incorporation of thymidine provoked by TGF-β, when compared with that caused by insulin. The delay observed was shorter than that reported by other authors for fibroblasts (41, 42) and smooth muscle cells (2). The difference may be due to the tumoral features of the L-929 cell line.

The addition of TGF-β provoked a mitogenic response proportional to the amount of added factor. This effect was reflected in an increase of up to 2.2 fold in the synthesis of DNA, and up to 1.7 fold in the synthesis of RNA; the synthesis of RNA reached a maximum with concentrations of TGF-β lower than 5 ng/ml (fig. 2A). No bimodal response to TGF-β was observed using a wide range of concentrations of this factor in contrast to what had been found with foreskin fibroblasts (2) and NRK fibroblasts (27). This response was also elicited by insulin (fig. 2B), the effect being noticeable even at concentrations as low as 0.5 ng/ml, within the physiological range. The concentrations of insulin were kept below those ordinarily used, to prevent non-specific binding by other low affinity receptors (22). The combination of insulin and TGF-β enhanced the incorporation of [^{3}H]-Thymidine and of [^{14}C]-Uridine, reaching the level provoked by fetal bovine serum with regard to the synthesis of DNA (fig. 3). When PDGF was tested on L-929 cells under the same experimental conditions, no response was obtained. Neither an increase in DNA (fig. 3) nor RNA (fig. 4) was observed. When PDGF was tested in combination with TGF-β, the effect of TGF-β by itself with regard to the synthesis of DNA was not modified (fig. 3).

Cooperation between TGF-β and insulin. — After 12 hours, when the incorpora-

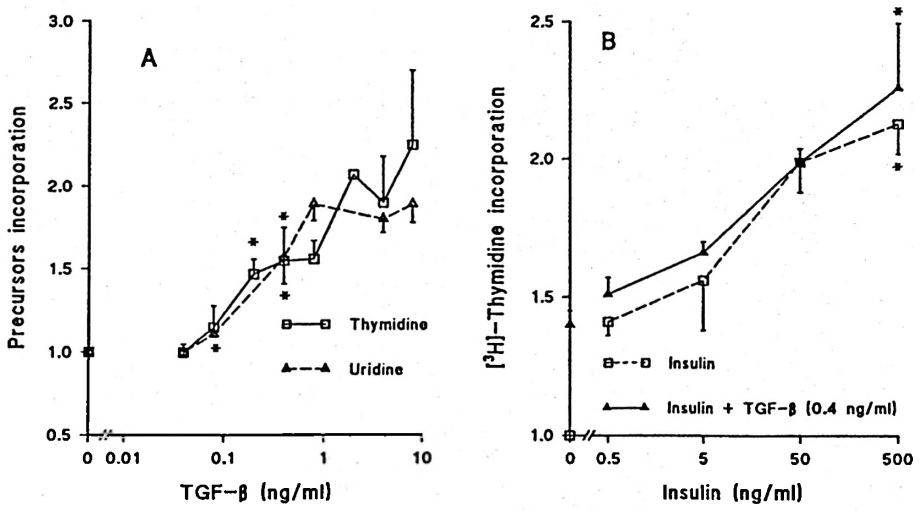

Fig. 2. *TGF-β increases the rate of thymidine or uridine incorporation in serum-free medium (A) but does not enhance insulin effect on the stimulation on the rate of thymidine incorporation in L-929 cells in serum-free medium after 18 h (B), fold of control.*
Cells were previously maintained in serum-free medium for 24 h [^{3}H]-Thymidine or [^{14}C]-Uridine was added 20 h (A) or 16 h (B) after TGF-β for a 2-h pulse. * $p<0.005$ as compared to no addition (Student's *t* test).

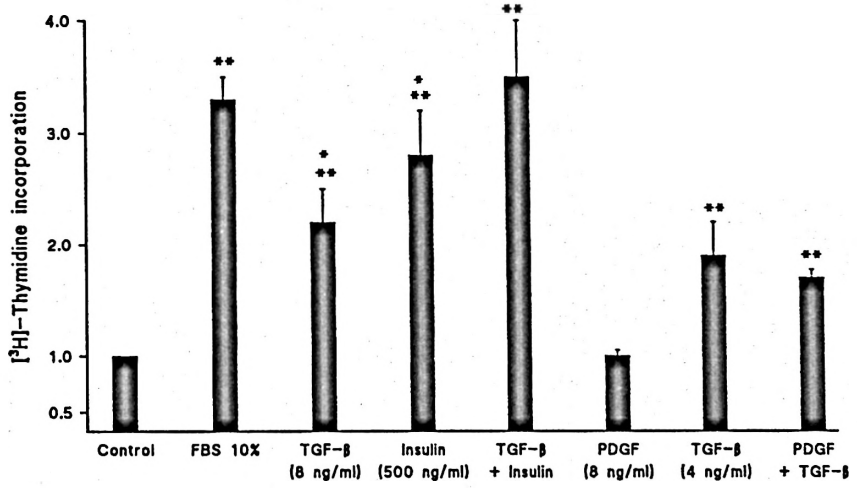

Fig. 3. *Effect of TGF-β, insulin and PDGF on the rate of thymidine incorporation in L-929 cells in serum free medium, fold of control.*
Cells were previously maintained in serum-free medium for 24 h [^{3}H]-Thymidine was added 20 h after TGF-β for a 2-h pulse. * $p<0.05$ as compared to TGF-β + insulin. ** $p<0.005$, as compared to control (Student's *t* test).

tion of thymidine was not affected by TGF-β, even at high concentrations (8 ng/ml), this factor did not increase the incorporation caused by 500 ng/ml of insulin (2.97 fold). The effect of the combined addition of TGF-β and insulin on the

synthesis of DNA after 18 hours is represented in fig. 2B. At that time, the effect caused by TGF-β alone had not reached its maximum value. TGF-β (0.4 ng/ml) affected only slightly the incorporation of thymidine over that caused by insulin alone. At 22 hours, TGF-β and

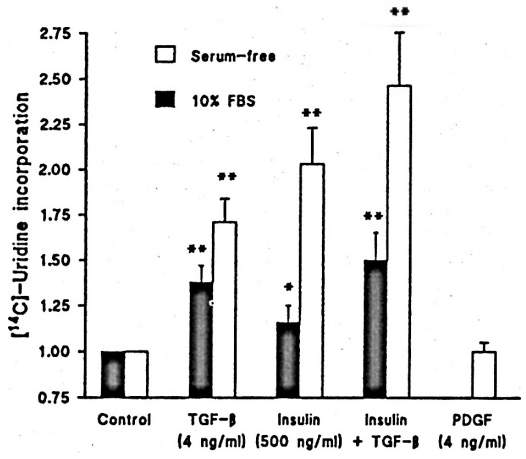

Fig. 4. *Effect of different factors on the rate of uridine incorporation, fold of control.*
Cells were previously maintained in serum-free for 24 h [^{14}C]-Uridine was added after the factors for a 2-h pulse. * $p<0.05$, ** $p<0.005$, as compared to control (Student's t test).

insulin together increased thymidine incorporation in L-cells reaching values similar to those obtained with 10 % FBS (fig. 3). These two factors affected also the synthesis of RNA in a similar pattern as that of DNA synthesis (fig. 4).

It has been postulated that in the Swiss 3T3 both TGF-β and insulin could coincide in their mechanism of action at a post-receptor level (4). However, insulin-like growth factors added together with TGF-β1 to chicken adipocyte precursor produce a greater than additive response (6). We have not found a clear potentiation of insulin stimulation after shorter times. A possible interpretation could be that the mitogenic signal for these two factors is operative through different pathways, later converging and affecting DNA synthesis. The addition of the two factors provoked a higher effect than each one separately, but without synergism.

Effect of TGF-β or insulin in the presence of FBS. — Serum contains substances, some of them unknown, which either have growth properties or are capable of modulating the activity of other growth factors (25, 33, 46). In order to study the effect of fetal bovine serum in the activity of TGF-β, the effect of TGF-β in L-cells at different concentrations of fetal bovine serum has been compared. Using as reference cells in the presence of the different concentrations of FBS without added factors, an increase in the concentration of FBS up to 10 % in the medium led to a decrease in stimulation in the synthesis of DNA provoked by TGF-β (table I). However, TGF-β increased, although slightly (0.3 fold), RNA synthesis (fig. 4), suggesting that TGF-β can bind to its receptors under these conditions. The breast-cancer cell line MCF-7 behaves in a different way to the growth stimulation by TGF-β depending on the presence or absence of serum (10). This seems to suggest that FBS contains, among others, TGF-β-like factors responsible for the growth of L-929 cells. These factors present in FBS could lead to a maximum mitogenic response. Another possibility is that an interaction of added TGF-β with other components of the FBS

Table I. *Effect of FBS concentration on the mitogenic response of L-cells to TGF-β.*
Results (mean ± SD) represent relative values referred to that in the absence of TGF-β considered as 1.

	[^{3}H]-Thymidine incorporation
Serum-free	1.9 ± 0.3**
1 % FBS	1.3 ± 0.2*
2.5 % FBS	1.2 ± 0.1*
5 % FBS	1.0 ± 0.1
10 % FBS	1.0 ± 0.1

* $p < 0.05$, ** $p < 0.005$, as compared to control.

could take place after the binding of TGF-β to its receptor leading to an inhibition of DNA synthesis. The different effects of TGF-β1 on collagen production by the embryonic palate mesenchymal cells in the presence or in the absence of serum may be due to an interaction with PDGF (40).

Modulation of the mitogenic action of TGF-β and insulin. — LEOF *et al.* (19) have shown that TGF-β induces the expression of the c-sis gene, encoding the sequence corresponding to PDGF-B in fibroblast AKR-2B. SOMA and GROTENDORST (42) have found that the stimulation of human primary fibroblasts takes place through the autocrine production of the chain PDGF-A, and MAJACK *et al.* (20) have shown that in vascular smooth muscle cells TGF-β induces the expression of PDGF-A. The mitogenic action of TGF-β in human fibroblast correlates with the expression of the PDGF receptor (51). It has also been reported that TGF-β stimulates the proliferation of mesangial cells (14). TGF-β is not mitogenic for NRK fibroblast and it is associated with the incapacity of TGF-β to affect either PDGF-AA production or expression of its receptor (47). The results here reported show that when TGF-β provoked a twofold increase in DNA synthesis, PDGF had no effect (fig. 3). This seems to indicate that TGF-β is not acting through PDGF. The effect of the latter would be secondary, i.e., not mediating a stimulation caused by TGF-β. Something similar could occur with other cell lines. BUTTERWITH and GODDARD (6) suggest that the PDGF is not responsible for the proliferation of chicken adipocyte precursor caused by TGF-β1. Since adenosine is known to potentiate the mitotic effect of PDGF (12, 18) experiments were carried out in order to ascertain if that nucleoside affected the response induced by TGF-β or insulin. Figure 5 shows that the presence of adenosine did not affect the synthesis of DNA elicited by either of these two factors. Adenosine by itself is a weak mitogen in this cell line. The mitogenic action of both TGF-β and insulin could share some point of activation with adenosine. If TGF-β elicits the synthesis of PDGF, which in an autocrine fashion is responsible for DNA synthesis, then the presence of adenosine should enhance the action of TGF-β.

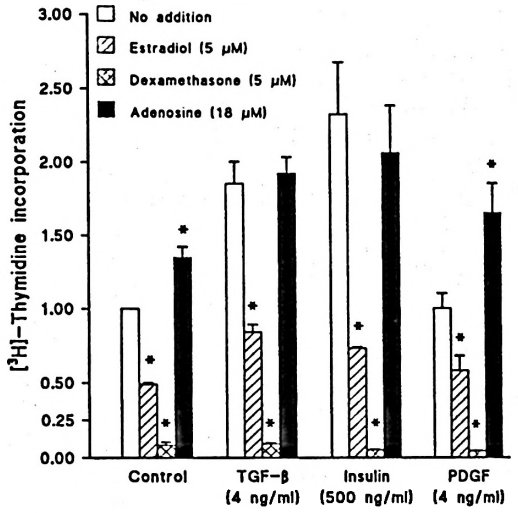

Fig. 5. *Effect of estradiol, adenosine and dexamethasone on the factor stimulation of the rate of thymidine incorporation in serum-free medium (fold of control).*
The assays were carried out in serum-free medium. * $p<0.005$, as compared to no addition (Student's *t* test).

Estrogens and dexamethasone are known to regulate the effect of the different growth factors (5, 28). Dexamethasone exerts a negative regulatory action on the pool of mRNA NGF in L-929 cells (50). The expression of TGF-β mRNA in mesenchymal cells is also regulated by estrogens and glucocorticoids (37). The results shown in figure 5 reveal that TGF-β abolished the inhibitory action of estradiol. Nevertheless, none of the three peptides studied could abolish the inhibitory action of dexamethasone. We assume that this glucocorticoid interferes with the mitogenic signal of both insulin and TGF-β.

Fig. 6. *Induction of c-myc mRNA (A) and/or β-NGF (B) by TGF-β and insulin.* Confluent cells were stimulated with either TGF-β (2 ng/ml) or insulin (700 ng/ml). At time intervals cells were collected and total RNA isolated. Lanes 1 to 4: Insulin, 2, 4, 6 and 12 h, respectively; lane 5: 10 % FBS, 12 h; lanes 6 to 9: TGF-β, 2, 4 and 6 h, respectively; lane 10: Control.

Effect of TGF-β and insulin on c-myc and β-NGF mRNA expression. — When the blot was hybridized with the c-myc probe (fig. 6A), a typical 2.4 kb band appeared on each lane, even on that of the control (lane 10). This proto-oncogen has been implicated in the proliferation and DNA synthesis; an alteration of its expression could be involved in the development of the tumour (34). The continuous expression of the c-myc could contribute to the tumorigenic feature of this cell line.

At 2 hours (lane 1) insulin caused a marked enhancement of the c-myc mRNA expression; however, the stimulation of the L-cells with TGF-β did not produce that effect until past 6 hours (lane 8). The difference in timing could be due to a different pattern of induction. This delay observed with TGF-β suggests that the induction in this case could be mediated by a growth factor, different from PDGF as indicated above.

L-929 cells synthesize β-NGF, and its biosynthesis is affected by several factors such as dexamethasone (49), vitamin D (50) and others. Figure 6B shows the hybridization of the blot with the ^{32}P-labelled β-NGF probe. The results revealed that insulin at 2 hours (lane 1) and TGF-β at 6 hours (lane 8) induced the expression of the β-NGF. However, it is worth noting that FBS had little effect on this expression. TGF-β has been found to be widely distributed in the nervous system (9, 21) and also that Schwan cells respond mitogenically to this factor (35). TGF-β elicited a response in the L-cells with an accumulation of β-NGF mRNA (fig. 6B), which, in turn, promotes neurite outgrowth (29, 30). Therefore, this factor could have an indirect neutrophic effect mediated by β-NGF.

TGF-β could act as an indirect mitogen in this cell line through an endogenous growth factor, different from PDGF, that seems to be the growth mediator in other mesenchimal cell lines (2, 19, 43). Nevertheless, β-NGF does not seem to be the mitogenic mediator of TGF-β because the

induction was transitory and occurred when that of the c-myc gene was taking place. The delay of several hours for β-NGF mRNA to accumulate with respect to insulin suggests that this response could also be mediated.

Acknowledgements

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Resumen

El TGF-β estimula la síntesis de DNA en un amplio intervalo de concentraciones en un medio sin suero y la insulina produce un efecto similar incluso a tiempos más cortos. Cuando se añaden conjuntamente, se consigue un efecto mitótico mayor que el producido por cada uno separadamente, aunque sin sinergismo. El PDGF, que no es mitogénico por sí mismo en esta línea celular, no aumenta la respuesta al TGF-β. En presencia de SBF ni el TGF-β ni la insulina estimulan la síntesis de DNA. Estos resultados sugieren que dos de los más importantes factores presentes en el SBF son la insulina y el TGF-β. La adenosina no modifica la respuesta mitogénica al TGF-β y a la insulina. Sin embargo, el PDGF estimula el crecimiento de las células L-929 en presencia de adenosina. Estos resultados indican que el TGF-β no estimula el crecimiento de las células L-929 a través de una producción autocrina de péptidos tipo PDGF en este modelo sin SBF. El TGF-β bloquea la respuesta inhibitoria de altas concentraciones de estradiol en las células L-929, pero no afecta a la inhibición producida por dexametasona. Tanto la insulina como el TGF-β aumentan la expresión del RNAm del c-myc y del β-NGF. Este efecto se manifiesta en tiempos más cortos cuando se adiciona insulina. La diferencia sugiere que la acumulación de RNAm causada por el TGF-β es mediada. El SBF ejerce poco efecto en la expresión de estos dos RNAm.

Palabras clave: TGF-β, Insulina, PDGF, Acción mitogénica.

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