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# INHIBITORY EFFECT OF TRANSFORMING GROWTH FACTOR-β ON EPIDERMAL GROWTH FACTOR-INDUCED PROLIFERATION OF CULTURED RAT AORTIC SMOOTH MUSCLE CELLS*

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Summary: This study was conducted to investigate the effect of transforming growth factor-β (TGF-β) on the proliferation of cultured rat aortic smooth muscle cells (SMCs). DNA synthesis, measured by the incorporation of [$^3$H] thymidine, and the cell number of monolayered SMCs were measured after incubation with TGF-β (1-100 ng/ml) in the presence or absence of epidermal growth factor (EGF;100 ng/ml). TGF-β alone did not affect DNA synthesis of SMCs. EGF significantly increased both DNA synthesis and cell number, while TGF-β inhibited the increase in both in a dose-dependent manner without accompanying the significant cellular damage. These results indicate that TGF-β exerts an inhibitory effect on the proliferation of cultured SMCs prvoked by EGF. 

Proliferation of vascular SMCs has been known to be an important process in the development of atherosclerosis (1,2). Some kinds of growth factors such as PDGF and EGF are suggested to be involved in this process, since these growth factors are released from the activated platelets (2,3, 4,5), and both are potent mitogens of vascular SMCs. Proliferation of SMCs in the atherogenic process is not infinite unlike malignant tumors and an inhibitory factor(s) might exist.

TGF-β was originally found as a peptide active in transforming normal cells into a neoplastic phenotype (6) and in stimulating anchorage-dependent cells to grow in an anchorage-independent condition (7,8,9). It could be a candidate for the inhibitory factor based on the evidence that

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Abbreviations: SMCs, smooth muscle cells; TGF-β, tranforming growth factor-β; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatic growth factor; MEM, minimal essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; PBS(-), phosphate buffered saline without $Mg^{++}$ and $Ca^{++}$; TCA, tricholoroacetic acid; [$^3$H]2-DOG, [$^3$H]2-deoxy-D-glucose

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TGF-β inhibits the proliferation of some kinds of cultured cells such as BSC-1 cells (10), monolayered NRK cells (11), rabbit renal proximal tubular cells (12), rat hepatocytes (13,14), and some neoplastic cells (11). The fact that platelets contain abundant TGF-β suggests that there may be biological effects on vascular cells. Actually, the inhibitory effect of TGF-β on the proliferation of vascular endothelial cells has previously been reported (15,16). The investigations into the effect of TGF-β on the proliferation of vascular SMCs, however, are very few. We thus studied the effect of TGF-β on the proliferation of vascular SMCs, and also the interaction between TGF-β and EGF on it.

## MATERIALS AND METHODS

### Chemicals

EGF was purchased from Collaborative Research (Lexington, MA, USA). TGF-β, which was purified from human platelets by Nakamura et al. (13), and Triton X-100 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [Methyl-$^3$H] thymidine (2.0 Ci/mmol in specific activity) and [$^3$H] 2-DOG (7.1 Ci/mmol in specific activity) were purchased from New England Nuclear (Boston, MA, USA). Ionomycin was a product of Carbiochem (La Jolla , CA, USA).

### Cell Culture

SMCs were isolated from the aorta of 5-week-old male Wistar rats according to the method described elsewhere (17). Briefly, small explants were aseptically cut from the media of thoracic aorta, placed on plastic dishes, and subjected to culturing in Medium 199 supplemented with 20% FCS in an atmosphere of 5% $CO_2$ and 95% air at 37$^0$C until the cells migrated from the explants. The explants were removed and culturing was continued until the cells became confluent. These confluent cells were then detached using a trypsin (0.125%)/EDTA (0.005%) solution, and subcultured in MEM supplemented with 10% FCS. These cells presented the "hill-and-valley" appearance considered to be characteristic of SMCs. Cells collected from the 8th to 10th passage were used for the experiments.

### Measurement of DNA Synthesis

Confluent SMCs were detached with trypsin/EDTA solution, and suspended in MEM with 10% FCS. Approximately 5000 cells were then inoculated onto 96-multiwell plates (0.32 cm$^2$/well; Nunc, Kamstrup, Denmark). The cells were incubated until they became subconfluent after approximately two days. To eliminate the growth accelerating effect of FCS, MEM with 0.01% FCS was substituted for the previous medium 24 hours prior to the experiment. TGF-β was added to the medium to give final concentrations of 1, 3.3, 10, 33 and 100 ng/ml, followed by the addition of EGF (100 ng/ml). Finally, one μCi/ml of [$^3$H]thymidine was added. After incubation for 36 hours, the medium was discarded and the cells were washed three times with ice-cold PBS(-), and subsequently incubated in ice-cold TCA (5%) for 20 minutes. After removal of TCA, the cells were lyzed with 50 μl of NaOH (0.5 N), and the cell lysate was titrated with the same volume of HCl (0.5 N). It was then transferred to the counting vial which contained 1 ml of Pico-Fluor 15 (Packard Instruments, Downers Grove, IL, USA). The radioactivity of the mixture was counted with a liquid scintillation counter (Aloka LSC 700, Tokyo, Japan), and was considered to be the index of the amount of DNA synthesis. These experiments with or without EGF were performed at the same time.

Smooth Muscle Cell Number Counting

SMCs were incubated in MEM with 0.01% FCS together with TGF-β (1-100 ng/ml) and EGF (100 ng/ml) using the same method as described in the measurement of DNA synthesis. After incubation for 36 and 72 hours, the cells were detached with 100 µl of trypsin/EDTA solution, and were then suspended in the diluent (CE-310; Toa, Kobe, Japan). The cell number was counted with an automatic cell counter (Toa CC-108, Kobe, Japan).

Estimation of Smooth Muscle Cell Injury

Smooth muscle cell injury was estimated based on the release of incorporated [$^3$H]2-DOG from the SMCs by the method originally developed for vascular endothelial cells (18). Briefly, SMCs were inoculated onto 96-multiwell plastic dishes and were cultured until confluency was reached. SMCs were incubated with [$^3$H]2-DOG (1 µCi/ml) for 18 hours at 37$^0$C. The cells were washed with Hanks' balanced salt solution containing 0.5% BSA. TGF-β dissolved in PBS(-) containing 0.025% BSA was incubated along with the SMCs at 37$^0$C for 1 hour. The radioactivities in the medium and in the cell lysate obtained by the treatment with 0.2 ml Triton X-100 (2%) were measured with a liquid scintillation counter (Aloka LSC-700, Japan). The specific release of [$^3$H]2-DOG from the SMCs was defined as 100x(A-C)/(B-C) (%), where A is the radioactivity in the cell medium treated by TGF-β, B is the total radioactivity in the medium and cell lysate of the treated cells, and C is the radioactivity in the control cell medium without being treated by TGF-β.

Statistics

The data were analyzed using the one-factor analysis of variance. If statistically significant effects were found, either Newman-Keuls' test or Duncan's test was performed to isolate the differences between the groups. A p value of less than 0.05 was considered to be significant. All data are presented in the text and figures as mean ± SEM.

## RESULTS

### Effects of EGF and TGF-β on DNA Synthesis of SMCs

As shown in the left panel of Fig. 1, TGF-β alone did not provoke any effects on the DNA synthesis of SMCs. As shown in the right panel of Fig. 1, EGF significantly increased the DNA synthesis compared to the control without EGF. TGF-β inhibited the increase in DNA synthesis evoked by EGF in a dose-dependent manner. DNA synthesis was decreased to the control level by the 33- and 100-ng/ml concentratons of TGF-β.

### Effects of EGF and TGF-β on Smoothe Muscle Cell Number

The effects of EGF and TGF-β on the SMC number after 36 hours of incubation are shown in the left panel of Fig. 2. As is evident, EGF significantly increased the SMC cell number and TGF-β inhibited the increase in the cell number in a dose-dependent manner. Similar and more apparent results were obtained after 72 hours of incubation as shown in the right panel of Fig. 2. The increase in the cell number evoked by EGF was inhibited to the control level by the 33- and 100-ng/ml concentration of TGF-β as in the DNA synthesis results.

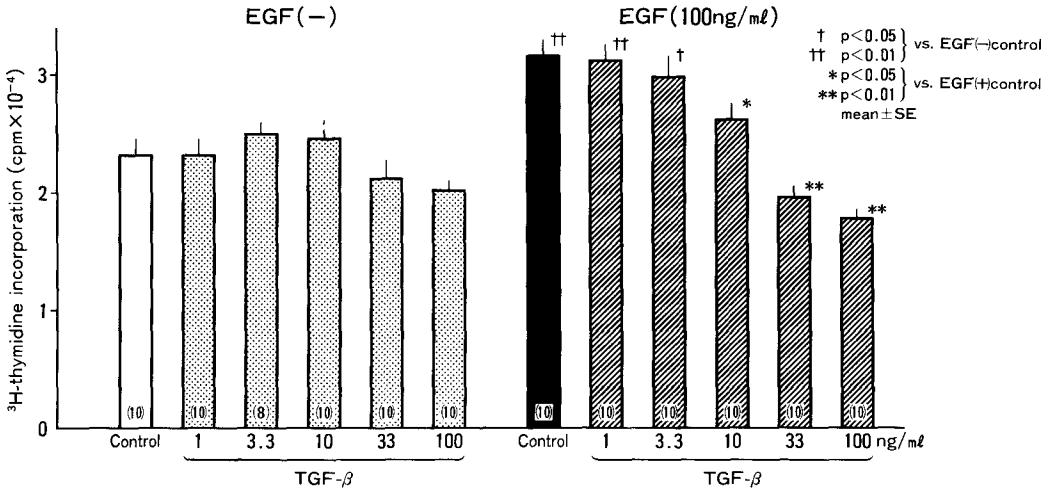


Fig. 1: The effects of TGF-β on DNA synthesis of SMCs. Left panel shows the effect of TGF-β alone, and right panel shows the effect of TGF-β on EGF-induced increase in DNA synthesis of SMCs. Number of experiments are shown in parentheses.

## Smooth Muscle Cell Injury

As shown in Fig. 3, the specific release of [$^3$H]2-DOG caused by TGF-β at various concentrations remained very low. In contrast, ionomycin ($10^{-5}$ M) and Triton X-100 (0.02%) caused significantly higher specific releases.

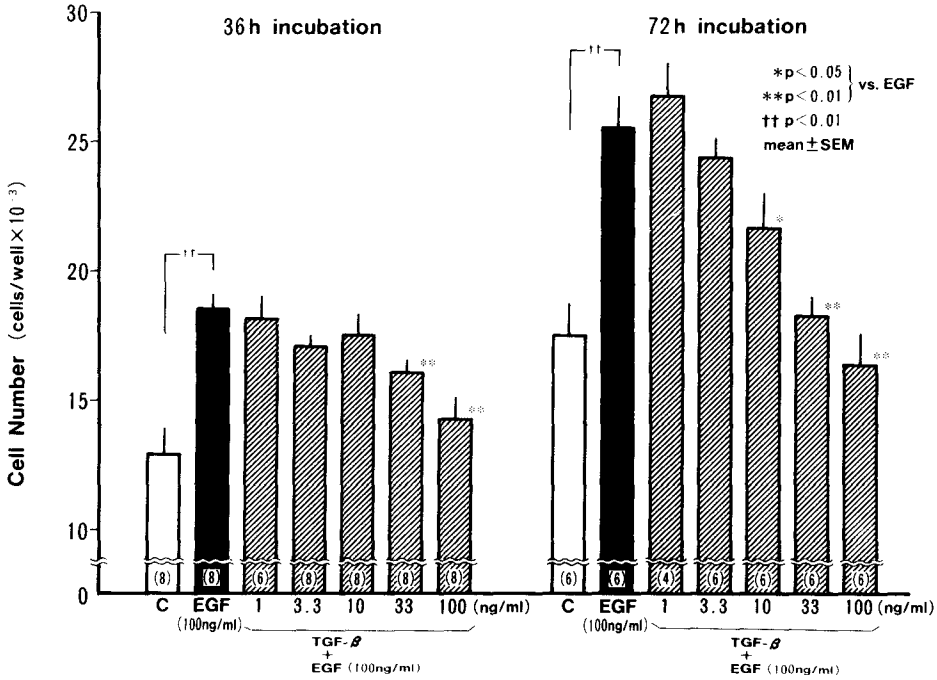


Fig. 2: The effect of TGF-β on the EGF-induced increase in cell number of SMCs. Left panel shows the result obtained after 36 hours of incubation, and right panel shows that obtained after 72 hours of incubation. Number of experiments are shown in parentheses.

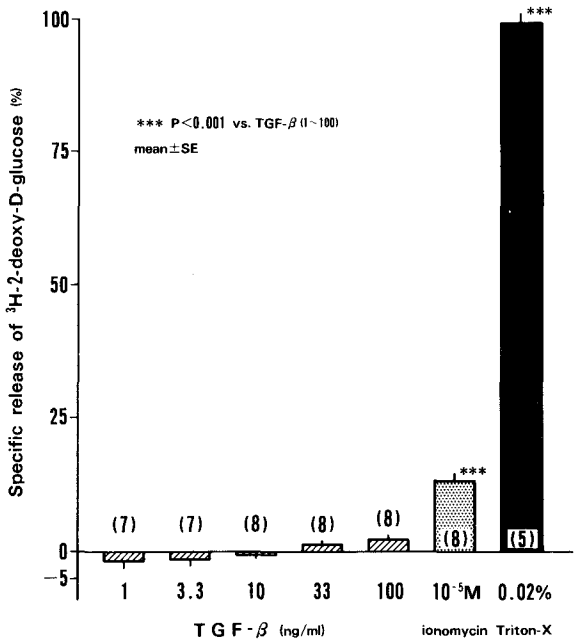


Fig. 3: The effects of TGF-β, ionomycin and Triton X-100 on the specific release of incorporated [$^3$H]2-deoxy-D-glucose from SMCs. Number of experiments are shown in parentheses.

## DISCUSSION

In the present study, we have shown that TGF-β inhibits the proliferation of vascular SMCs stimulated by EGF, since TGF-β inhibited the increase both in DNA synthesis and in the cell number. This result is compatible with that reported by Assoian and Sporn who observed that TGF-β suppressed the increase in the cell number of bovine aortic SMCs induced by EGF (19). The inhibitory effect was not due to a non-specific phenomenon provoked by cellular damage, since TGF-β did not provoke any significant release of incorporated [$^3$H]2-DOG. Interestingly, TGF-β alone did not provoke any effects on DNA synthesis of SMCs in the absence of EGF. This observation suggests that the effect of TGF-β is not a function of the peptide itself. Rizzino et al. (20) have shown that the biological effect of TGF-β on the anchorage-independent proliferation of NRK cells varies according to the combination of concomitant growth factors including PDGF , FGF and EGF, supporting the idea that TGF-β itself has no proper biological function.

The cellular mechanism of the inhibitory action of TGF-β has not been fully understood. It is unlikely that TGF-β competitively binds to EGF receptors, because there is no homologous primary structure between TGF-β and EGF. Moreover, no cross-reactivity between TGF-β and EGF receptors

was found unlike the case of TGF-α (21). Based on the evidence that TGF-β is capable of inhibiting the proliferation of cells provoked by many growth factors, such as FGF (15) and HGF (16), it is possible that TGF-β could act on the common pathway of post-receptor signal transduction of the peptide growth factors. Actually, Riedel et al. (22) suggested that the mechanism of post-receptor signal transduction might be common for growth factors. This was suggested by showing that insulin can activate the tyrosine kinase activity of EGF receptor through the chimeric receptor having the extracellular domain of insulin receptor and having the transmembrane and cytoplasmic domains of EGF receptor. However, the explanation remains to be determined for the reversed biological effect of TGF-β on the cells such as NRK cells according to the culturing condition (11).

The present study clearly shows that TGF-β has an inhibitory action on the proliferation of vascular SMCs. A higher concentration of TGF-β than those reported for other kinds of cultured cells was required in this study for the inhibition. For example, Nakamura et al. (13) reported that the proliferation of hepatocytes induced by insulin and EGF was completely suppressed by the addition of only 1.6 ng/ml of TGF-β. This may arise from the different cell types. Another possibility is the fact that cells used in the present study were obtained from explants of aortic media. The cells from explant culturing may have a higher potency of growth.

EGF has been known to be a potent mitogen for vascular SMCs as has also been demonstrated in this study. Oka and Orth have shown that α granules of platelets contain considerable amounts of EGF (5). Since the proliferation of SMCs is important in the atherogenic process (1,2), it is highly possible that EGF plays an important role in the development of atherosclerosis. This study has demonstrated that TGF-β, which has been shown to be released from the α granules of platelets (23), has an inhibitory action on the proliferation of vascular SMCs provoked by EGF. The result suggests the possible role of TGF-β as a negative modulator for the development of atherosclerosis. Further studies will be required to elucidate the role of TGF-β in athrogenesis in vivo.

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