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INJURY INDUCED EXPRESSION OF TGF-ß1 mRNA IS ENHANCED BY EXOGENOUSLY APPLIED TGF-ßS

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We have analysed and compared, by in situ hybridisation, the effects of exogenously applied TGF-ßs on expression of endogenous TGF-ß mRNAs in partial thickness thermal wounds in old and young mice. Although injury induced the expression of TGF-ß1 mRNA in the epidermis and dermis at the wound margins, expression of TGF-ß2- or TGF-ß3-mRNA was not detected. Biopsies taken 24 hours following injury revealed a focally clustered distribution of TGF-ß1 hybridisation signals in the dermis, the number of positive cells and expression levels being reduced in old mice. Topical application of all three TGF-ß isoforms enhanced TGF-ß1 mRNA expression in the dermis of old and young mice. In biopsies taken three days following injury, TGF-ß1 hybridisation signals were most prominent in the regenerating epidermis although at this timepoint differences in expression levels between treated and non-treated animals were less pronounced.

Cutaneous injury initiates a cascade of events which normally results in complete healing of a wound. Major events in the repair process include blood coagulation, platelet aggregation and degranulation, recruitment of inflammatory cells, proliferation of connective tissue cells, remodelling of extracellular matrix, epithelial cell migration and proliferation leading to re-epithelialization of the wound (reviewed in 1). Transforming Growth Factor-ßs (TGF-ßs) have been implicated as important mediators of wound healing because of their potent effects on macrophage and fibroblast recruitment (2,3), their strong stimulatory effects on the formation of extracellular matrix proteins (4,5), and their well documented angiogenic effects (6,7,8,9). Numerous in vivo studies have shown that the local application of TGF-ß

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isoforms can enhance the repair process in animal wound healing models. In this respect TGF-ß appears to be especially effective in stimulating repair under conditions in which the normal healing processes are either artificially or naturally impaired (reviewed in 10). However, our present knowledge of the regulation and expression of endogenous TGF-ßs in normal skin and during the wound healing process is relatively limited. TGF-ß1 mRNA is induced in the porcine dermis following wounding and expression levels are enhanced by local application of TGF-ß1 protein (11,12). A recent immunohistochemistry study has provided evidence for increased secretion of TGF-ß1 by migrating keratinocytes at the margins of pig and human skin wounds (13).

The aim of the present study was to compare, by in situ hybridisation, mRNA expression of the three mammalian TGF-ß isoforms in partial thickness thermal wounds on young and old mice following topical application of TGF-ß1, -ß2, and -ß3 or placebo treatment.

MATERIAL AND METHODS

Growth factors: Recombinant human TGF-ß1 was obtained from IDS (Washington, U.K), recombinant human TGF-ß2 and TGF-ß3 were from Ciba-Geigy (Basel, Switzerland). All TGF-ßs were formulated in a viscous vehicle buffer solution (0.5% w/v hydroxypropylcellulose in 10mM histidine, 140mM NaCl, pH 7.4).

Wounding, treatment and collection of biopsy specimens: Partial thickness thermal wounds were induced on the dorsal thorax of anaesthetised young (3 month) and old (18 month) C57BL/6 mice by means of a brass block ($1cm^2$) equilibrated to 80^oC and pressed on the depilated skin for 10 seconds. The blister roof was removed 1 hour later and wounds were treated by topical application of 1 µg recombinant TGF-ß1, TGF-ß2, TGF-ß3 or with vehicle only. Skin biopsy specimens were collected 6 hours, 24 hours and 72 hours following injury.

In situ hybridisation: Biopsies were fixed for 24 hours at 4^oC in a freshly prepared solution of 4% paraformaldehyde in PBS and paraffin embedded. 6µm sections were hybridized with [^{35}S]-labeled riboprobes as described (14). The TGF-ß riboprobe templates were 339 nucleotide long fragments, subcloned into pGEM5 (Promega) and corresponded to the c-DNA sequences encoding the mature forms (plus stop codon) of human TGF-ß1, TGF-ß2, and TGF-ß3. Specificities of all riboprobes were checked by Northern blot analysis and "sense" probes were used as negative controls. Exposure times were 2 weeks. Sections were stained with Haematoxylin.

RESULTS

Expression of TGF-ß mRNAs in intact skin and 6 hours following wounding. Specific TGF-ß1, TGF-ß2, and TGF-ß3 expression was not detected in non-wounded mouse skin (data not shown) and only very weak

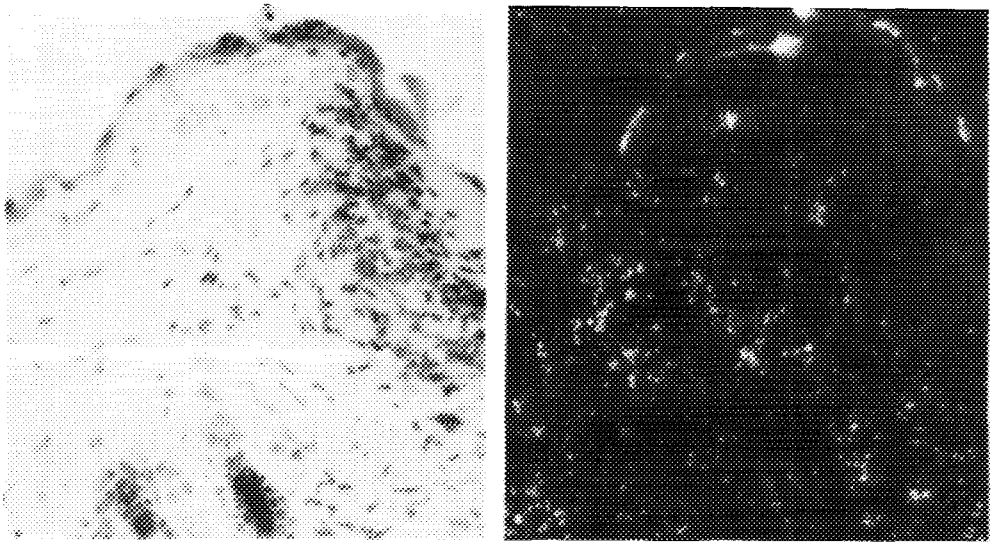

Fig.1. In situ localization of TGF-ß1 mRNA at the edge of a partial thickness thermal wound 6 hours following injury of a young mouse. Left) brightfield illumination; right) darkfield illumination (showing reflected light from silver grains). Only very weak TGF-ß1 hybridisation signals are visible in the dermis at the wound margin.

TGF-ß1 hybridisation signals were found in the dermis of all skin biopsies taken 6 hours following wounding (**Fig.1**).

Expression of TGF-ß mRNAs 24 hours following wounding. A focally clustered distribution of TGF-ß1 hybridisation signals was observed in the dermis at the wound margin (**Figs.2 & 3**). Biopsies from non-treated young mice (**Fig.2A**) revealed higher levels of TGF-ß1 hybridisation signals than those from non-treated old mice (**Fig.3A**). Both in young and old mice the number of TGF-ß1 positive cells and the overall extent of expression within the wounded dermis increased after topical treatment of the wounds with TGF-ßs (**Figs. 2 & 3**). All three TGF-ß isoforms turned out to be potent stimulators of TGF-ß1 expression. TGF-ß treatment of wounds in old mice increased the level of TGF-ß1 expression to a level (**Fig.3B**) which was higher than that observed in non-treated young mice (**Fig.2A**). A further stimulation of TGF-ß1 expression was visible in wounds of young mice following topical application of TGF-ßs (**Fig.2B-D**). Specific expression of TGF-ß2 and TGF-ß3 mRNAs was not detected in any of the wound biopsies taken from untreated or TGF-ß-treated wounds.

Expression of TGF-ß mRNAs 72 hours following wounding. In comparison to the 24-hour biopsies, higher levels of TGF-ß1 mRNA expression were observed in the regenerating epidermis after 72 hours (**Fig.4**). At this time however, TGF-ß1 mRNA transcripts were more uniformly distributed in the dermis. At 72 hours post wounding differences in TGF-ß1

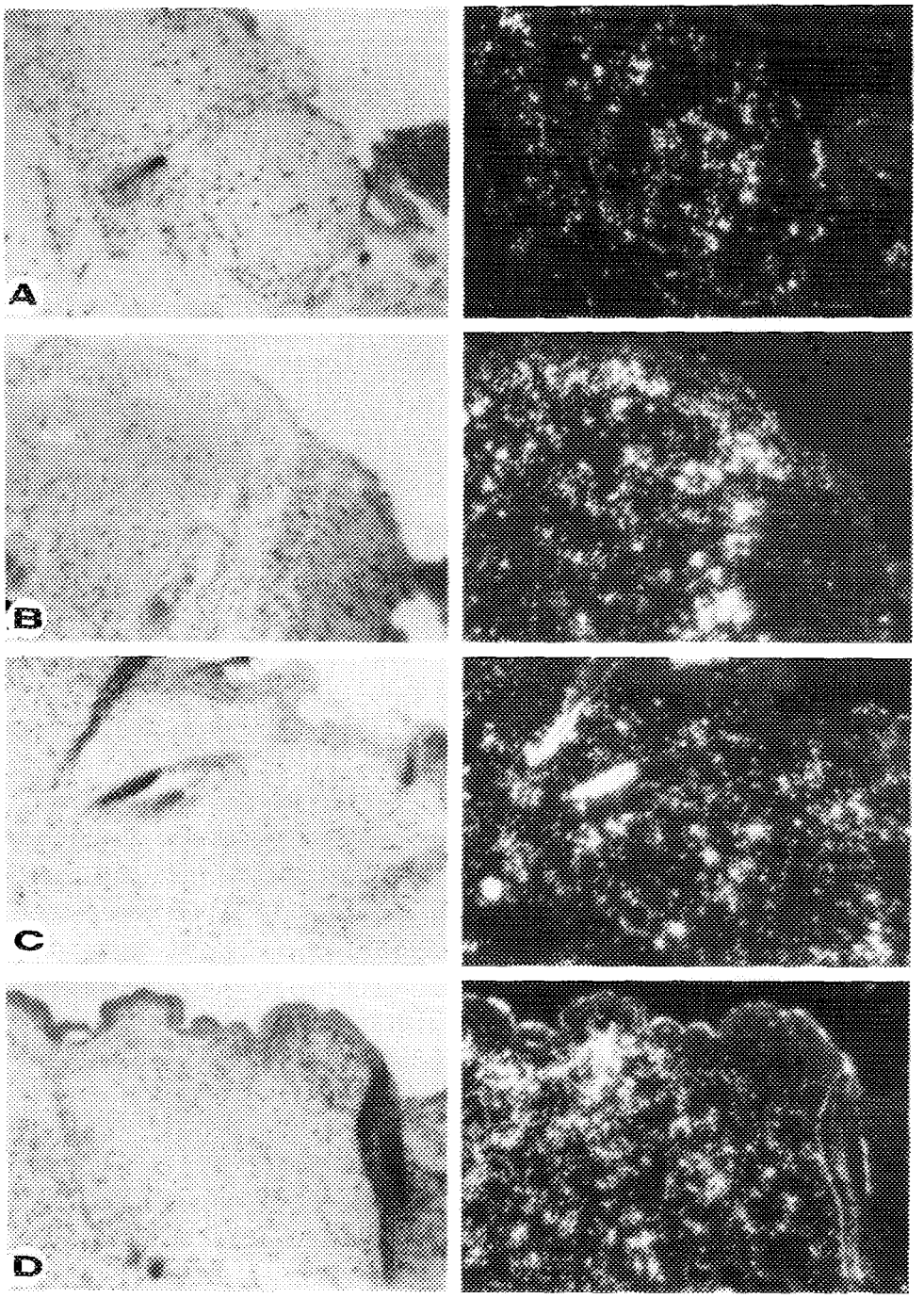

Fig.2. In situ hybridisation showing TGF-ß1 mRNA expression in biopsies taken 24 hours following wounding of young mice treated with: A) vehicle only; B) 1µg TGF-ß1; C) 1µg TGF-ß2; D) 1µg TGF-ß3. Left) brightfield illumination; right) darkfield illumination. A focally clustered distribution of TGF-ß1 hybridisation signals is visible in all biopsies at the wound margins. The number of TGF-ß1 positive cells and the overall extent of expression is increased in TGF-ß treated mice.

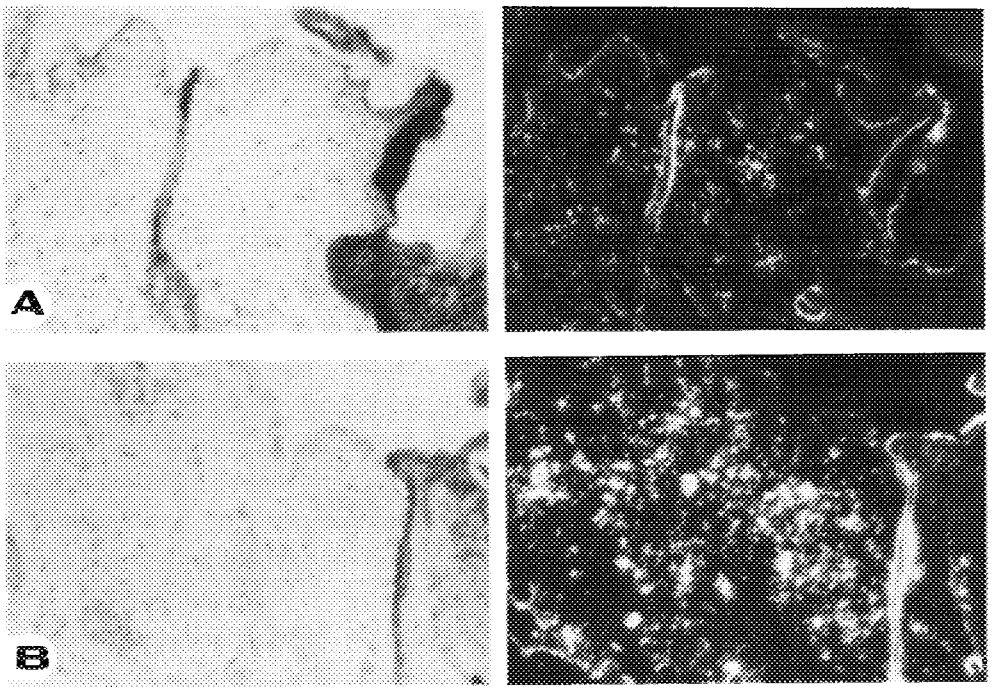

Fig.3. In situ hybridisation showing TGF-ß1 mRNA expression in biopsies taken 24 hours following injury of old mice treated with: A) vehicle only; B) 1µg TGF-ß3. Left) brightfield illumination; right) darkfield illumination. Increased levels of TGF-ß1 hybridisation signals are visible at the wound margin of a TGF-ß3 treated mouse.

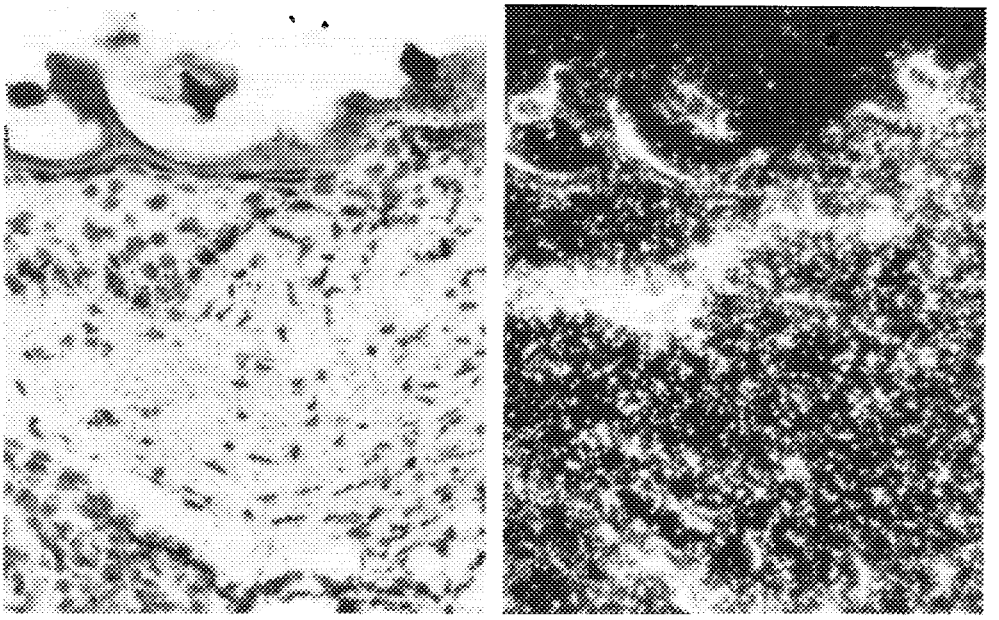

Fig.4. In situ localisation of TGF-ß1 mRNA at the edge of a partial thickness thermal wound 3 days following injury of a young mouse. Left) brightfield illumination; right) darkfield illumination. TGF-ß1 hybridisation signals are strongest in the basal cell layers of the regenerating epidermis.

expression levels between treated and non-treated animals were less pronounced and TGF-ß2 and TGF-ß3 hybridisation signals were again not detected.

DISCUSSION

The findings of the present study suggest that TGF-ß1 mRNA expression is decreased in wounds of old animals, where the wound healing status is known to be naturally impaired (9). Cox et al. (15) have shown that topical application of recombinant or natural TGF-ß2 enhanced and accelerated wound healing responses in old rodents to levels observed in non-treated young animals. In most instances a single application of TGF-ß2 was sufficient to obtain a full healing response. Our observations also provide evidence that exogenously applied TGF-ßs stimulate the expression of endogenous TGF-ß1, probably via an enhanced recruitment and/or activation of wound monocytes/macrophages. This finding is in agreement with the reported enhancement of TGF-ß1 expression in porcine skin wounds following treatment with TGF-ß1 (11,12). Salomon et al. (16) have shown that a restoration of wound healing in doxorubicin-treated animals, which also show reduced TGF-ß expression levels at the wound site, can be simply achieved by topical application of TGF-ß1 thereby providing further evidence for the efficacy of TGF-ß isoforms to heal impaired wounds.

Induction of TGF-ß1 expression in the dermis may be an important prerequisite for restoration of dermal matrix or granulation tissue formation. In addition, an induction of TGF-ß1 expression in epidermal cells at the edge of a wound may be an important signal for re-epithelialization to occur. This second idea is supported by several findings since TGF-ß1 has been shown to switch keratinocytes from a differentiating- to a regenerative-phenotype (17); to stimulate keratinocyte motility in vitro (18), and in ex-vivo biopsies (19); and to upregulate the production of fibronectin (18), an essential component of the provisional matrix over which epidermal cells need to traverse during the re-epithelialization process.

TGF-ßs are potent inhibitors of epithelial cell proliferation although these growth factors may stimulate proliferation of some mesenchymal cells via induction of Platelet-Derived Growth Factor (PDGF) (reviewed in 20). Normal skin epithelial cells do not constitutively express PDGF or PDGF-receptors although an induction of both proteins has been shown to occur following injury (21). TGF-ß1 may stimulate proliferation of keratinocytes at the wound margins indirectly, via an alternative pathway involving PDGF, thereby providing a plausable explanation for the thickening of the regenerative epidermis at the wound edge.

At the mechanistic level, cellular resposes to wounding include induction of AP-1 regulated genes (22) and autoinduction of TGF-ß1 has been shown to be mediated by the AP-1 complex (23). TGF-ß1 which is released from degranulating platelets and activated macrophages and is present in an active form in wound fluid (24-26) may therefore trigger a cascade of inductive events resulting in TGF-ß1 expression in dermal and epidermal cells at the wound site via an AP-1 mechanism.

From the findings of the present study we suggest the hypothesis that an induction of TGF-ß1 expression in dermal and epidermal cells is essential for normal wound healing to proceed. A stimulation of endogenous TGF-ß1 expression in wound tissues, e.g., by the addition of exogenously applied TGF-ßs, may therefore offer a promising therapeutic approach for the treatment of chronic, non-healing wounds.

REFERENCES

1. Richard, A.F., Clark, M.D., and Denver, C.O. (1985) J. Am. Acad. Dermatol. **13**, 701-725.
2. Postlethwaite, A.E., Keski-Oja, J., Moses, H., and Kang, A. (1987) J. Exp. Med. **165**, 251-256.
3. Wahl, S.M., Hunt, D.A., Wakefield, L.M., McCartney, F.N., Wahl, L.M., Roberts, A.B., and Sporn, M.B. (1987) Proc. Natl. Acad. Sci. USA **84**, 5788-5792.
4. Sporn, M.B., Roberts, A.B., Shull, J.H., Smith, J.M., and Ward, J.B. (1983) Science **219**, 1329-1331.
5. Ignotz, R. and Massaque,J. (1986) J. Biol. Chem **261**, 4337-4345.
6. Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauci, A.S. (1986) Proc. Natl. Acad. Sci. USA **83**, 4167-4171.
7. Leibovic, S.J., and Wiseman, D.M. (1988) In Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications, pp. 131-145. Alan R. Riss, New York.
8. Madri, J.A., Pratt, B.M., and Tucker, A. (1988) J. Cell. Biol **106**, 1375-1384.
9. Cox, D.A. Kunz, S., Cerlett,i N., McMaster, G.K., and Bürk, R.R. (1992) In Angiogenesis (R. Steiner, P.B. Wiesz and R. Langer, Eds), pp. 287-295. Birkhauser Verlag, Basel.
10. Martin, P., Hopkinson-Woolley, J. and McCluskey, J. (1992) Progress in Growth Factor Research **4**, 25-44.
11. Quaglino, D., Nanney, L.B., Kennedy, R., and Davidson, J. (1990) Lab. Invest. **63**, 307-319.
12. Quaglino, D., Nanney, L.B., Ditesheim, J.A., and Davidson, J.M. (1991) J. Invest. Dermatol. **97**, 34-42.
13. Kane, C.J., Hebda, P.A., Mansbridge, J.N. and Hannawalt, P.C. (1991) J. Cell. Physiol. **148**, 157-173.
14. Schmid, P., Cox, D.A., Bilbe, G., Maier, R., and McMaster, G.K. (1991) Development **111**, 117-130.

15. Cox, D., Kunz, S., Vaxelaire, J., Cerletti, N., McMaster, K.K., and Bürk R.R. (1993) In Proceedings of the 2nd European Conference on Advances in Wound Management (K.G. Harding, G. Cherry, C. Dealey and T.D. Turner, Eds.) pp. 135-139. McMillan, London.
16. Salomon, G.D., Kasid, A., Bernstein, E., Buresh, C., Director, E., and Norton, A. (1990) Surgery **108**, 318-323.
17. Mansbridge, J.N. and Hanawalt, P.C. (1988) J. Invest. Dermatol. **90**, 336-341.
18. Nickoloff, B.J., Mitra, R.S., Riser, B.L., Dixit, V.M., and Varani, J. (1988) Am. J. Pathol. **132**, 543-551.
19. Hebda, P.A. (1988) J. Invest. Dermatol. **91**, 440-445.
20. Lyons, R.M., and Moses, H.L. (1990) Eur. J. Biochem. **187**, 467-473.
21. Antoniades, H.N., Galanopoulos, T., Neville-Golden, J., Kiritsy, C.P., and Lynch, S.E (1991) Proc. Natl. Acad. Sci. USA **88**, 565-569.
22. Sosnowski, R.G., Feldman, S., Feramisco, J.R. (1993) J. Cell. Biol. **121**, 113-193.
23. Kim, S-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K.Y., Sporn, M.B., Karin, M., and Roberts, A.B (1990) Mol. Cell. Biol. **10**, 1492-1497.
24. Assoian, R.K. and Sporn, M.B. (1986) J. Cell. Biol. **102**, 1712-1733.
25. Rappolee, D.A., Mark, D., Banda, M.A., and Werb, Z. (1988) Science **241**, 708-712.
26. Grotendorst, G.R., Grotendorst, C.A., and Gilman, T. (1988) In Growth Factors and Other Aspects of Wound Healing, pp. 47-54. Liss, New York.