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Expression of Growth Factor and Receptor mRNAs in Skin Epithelial Cells Following Acute Cutaneous Injury

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We report that acute injury induces the expression of selective growth factor and growth factor receptors in the epithelial cells of the wounded tissue. **In situ** ***hybridization analysis of skin biopsy specimens obtained after cutaneous injury in swine demonstrated the induction of the expression of transforming growth factor-α, its receptor, epidermal growth factor-R, acidic fibroblast growth factor, and basic fibroblast growth factor messenger RNAs in the skin epithelial cells of the wounded tissue. There was no significant expression in the epithelial cells of control, uninjured tissues. The expression levels were maximal during the period of active tissue repair (1 to 5 days after injury) and were totally suppressed upon the healing of the wounded tissues. In contrast, insulinlike growth factor-I, (IGF-I), IGF-I receptor, and IGF-II receptor messenger RNAs were expressed in the epithelial cells of both the control, uninjured tissues and in tissue specimens obtained after injury. There was no significant expression of IGF-II messenger RNA in the epithelial cells before or after injury. It seems that injury induces the coordinated expression of selective growth factor and growth factor receptor genes whose products contribute to the regulation of the complex processes involved in tissue repair and remodeling. (Am J Pathol 1993, 142:1099–1110)***

Normal wound healing involves a series of events that result in connective tissue regeneration and epithelial cell migration and proliferation leading to the healing and reepithelialization of the wounded tissue. These processes are regulated by growth factors that have the ability to stimulate the proliferation and migration of responsive cells at the site of injury and the production of matrix components, including collagen synthesis. There are several reports on the successful application of exogenous growth factors for the healing of experimental wounds in a variety of animal models.[1–9] The source of endogenous growth factors controlling the normal wound healing processes *in vivo* is not clearly understood. It has been assumed that circulating platelets and inflammatory cells provided growth factors at the site of injury. Growth factors such as the platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) have been shown to be stored in platelets and to be produced by activated macrophages. Other growth factors, such as the insulinlike growth factors (IGFs) may be available to the wounded tissue from circulating blood. Recent studies suggested that the major and sustained source of growth factors during wound healing is provided by cells adjacent to the site of injury and by newly proliferating cells, including epithelial cells and connective tissue fibroblasts. A striking example is provided from studies on the expression of the PDGF-2, or B, chain of human PDGF and its receptor, the PDGF receptor β (PDGF-R β) following acute cutaneous injury.[10] The PDGF-2(B) chain is encoded by the simian sarcoma virus oncogene v-*sis* and by its cellular homologue, the c-*sis* protooncogene (reviewed in ref. 11). Acute cutaneous injury induced the strong expression of both c-*sis*/PDGF-2(B) and PDGF-R β messenger (m)RNAs in the epithelial cells at the site of injury. Connective tissue fibroblasts at the wound site also expressed c-*sis*/PDGF-2(B)

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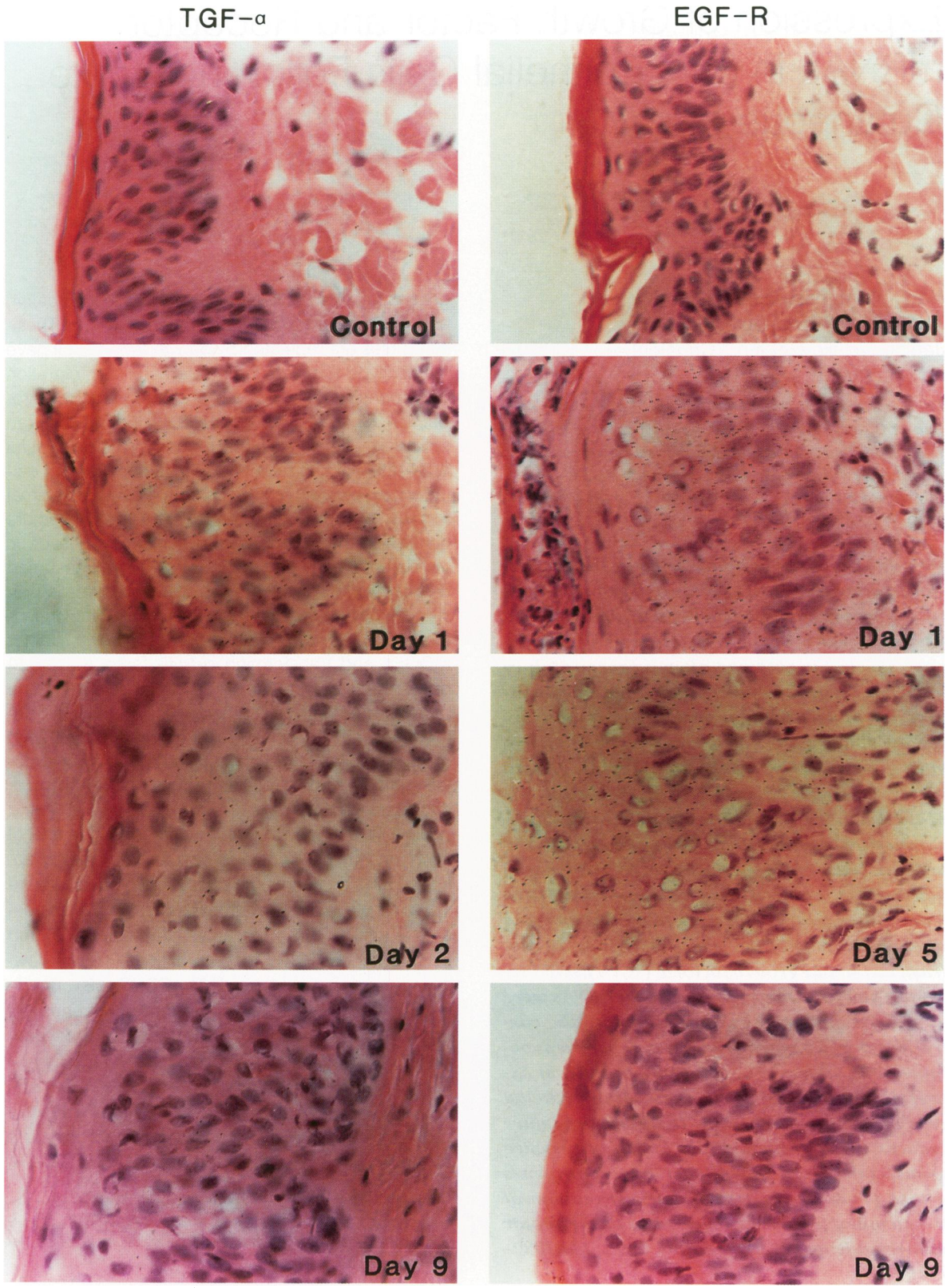

Figure 1. *Expression of TGF-α and EGF-R mRNAs in skin epithelial cells before and after acute cutaneous injury. There is no significant expression of TGF-α or EGF-R mRNAs in the epithelial cells of control, uninjured tissues. Messenger RNA expression can be seen within 1 day of injury and persists for about 5 days, declining to background levels by day 9. Original magnification: ×630.*

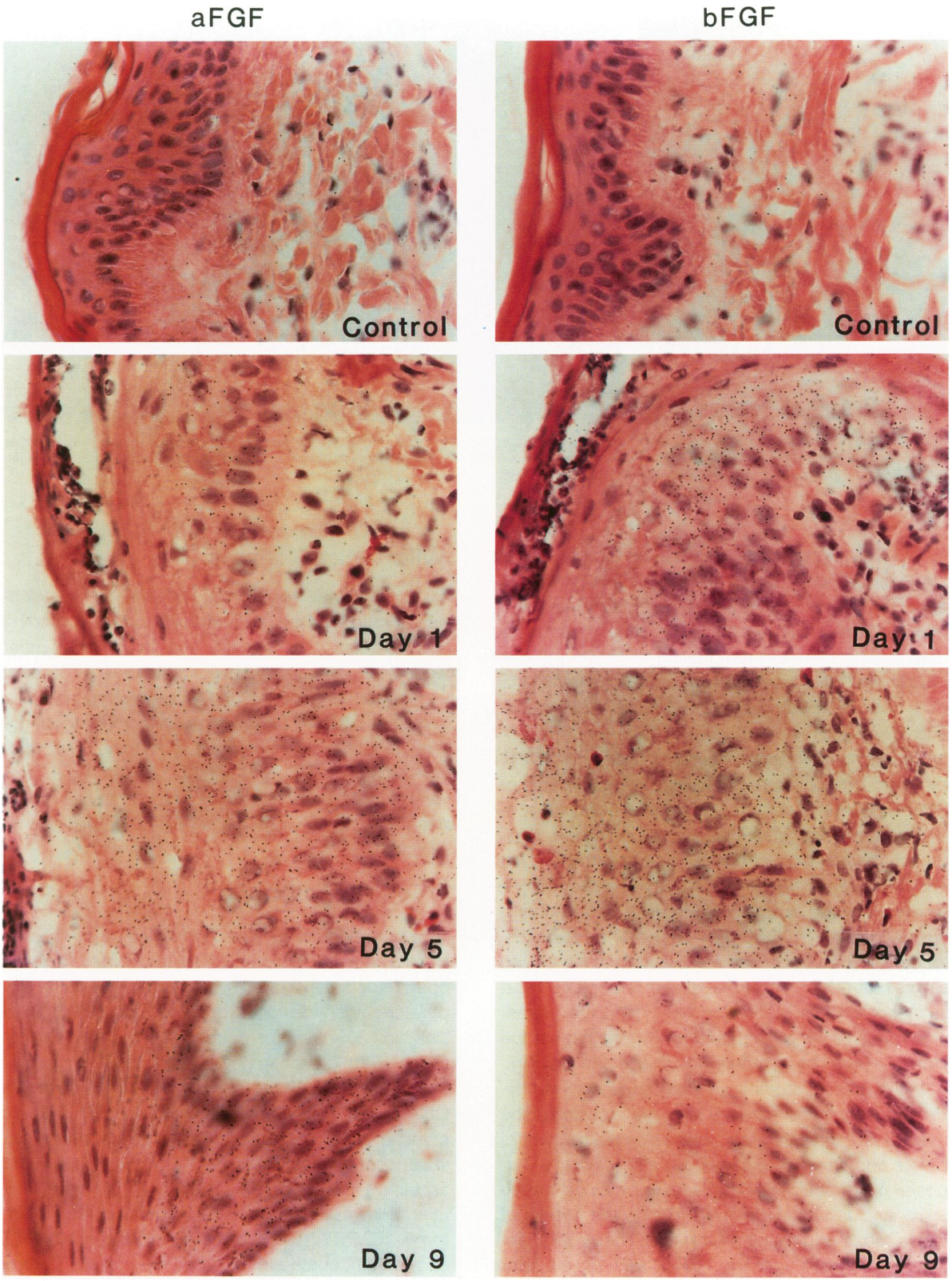

Figure 2. *Expression of aFGF and bFGF mRNAs in skin epithelial cells before and after cutaneous injury. There is no significant expression of aFGF or bFGF mRNAs in the epithelial cells of control, uninjured tissue. In both cases expression in the epithelial cells can be seen within 1 day of injury. The expression of aFGF mRNA increased gradually to day 9 and was completely suppressed by day 17. The expression of bFGF mRNA remained strong for about 5 days, declined by day 9, and was completely suppressed by day 17 after injury. Original magnification: ×630.*

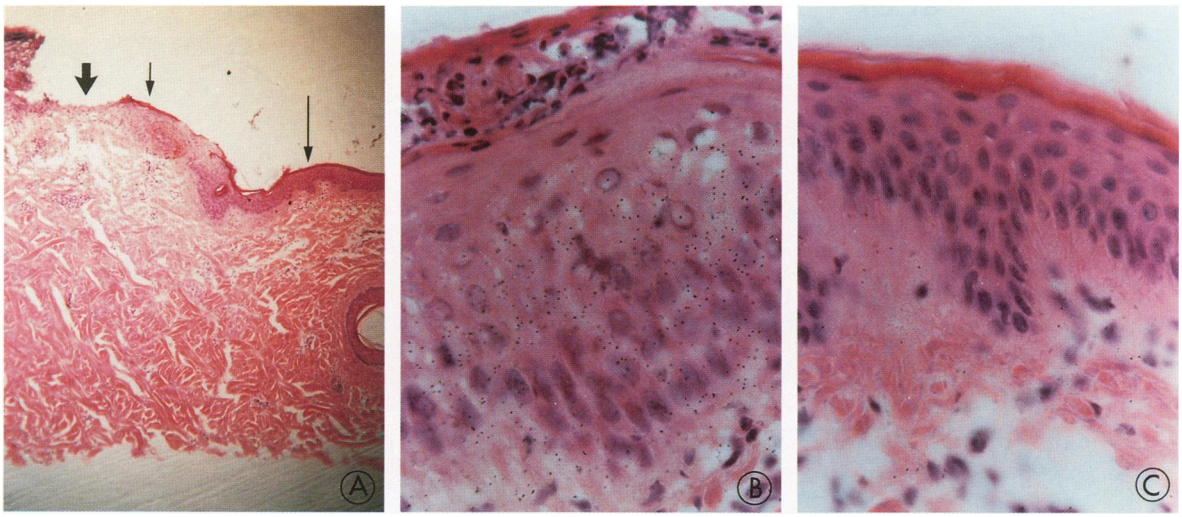

Figure 3. *The regions adjacent and distal to the injured site (bold arrow) are shown in* **(A)**. *Expression of EGF-R mRNA is seen at day 1 after injury in the epithelium adjacent to the injured site* **(B)** *(short arrow). There is no significant expression in the distal region of the intact skin epithelium* **(C)** *(long arrow). Original magnification:* **A:** ×63; **B, C:** ×630.

mRNA. The expression levels were correlated with the stage of wound repair, being highest during the time of rapid cell proliferation and matrix synthesis and declining and suppressed at the time of complete reepithelialization and remodeling.[10] These findings in epithelial cells were not expected, because these cells normally do not express c-*sis*/PDGF-2(B) mRNA and PDGF-R β mRNA. Similarly, connective tissue fibroblasts normally express only receptors to PDGF, but they do not express PDGF. The expression of PDGF and its receptor in skin epithelial cells and connective tissue fibroblasts reflects the presence of autocrine mechanisms contributing to cell proliferation and tissue regeneration. Other reports also provided evidence for the expression of growth factors during wound healing.[12–15] Studies based on immunohistochemistry have shown the expression at the wound site of TGF-β,[16,17] epidermal growth factor (EGF) and its receptor (EGF-R),[18,19] and IGF-1.[20–22] The expression levels of the growth factors were highest at the time of active tissue repair, declining upon healing.

In the present studies we have applied *in situ* hybridization to investigate the *in vivo* expression of the major known growth factors in the skin epithelial cells before and after acute cutaneous injury. For this purpose, skin biopsy specimens were obtained before injury and at various intervals during the course of tissue repair and reepithelialization. The growth factors investigated include TGF-α and its receptor (EGF-R); acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF); IGF-I and its type I receptor (IGF-I R); and IGF-II and its type II receptor (IGF-II R).

Materials and Methods

Total excisional skin biopsy specimens were obtained from normal, uninjured skin before wounding (control), daily for 9 days and at 17 days after wounding. Partial thickness wounds (10 × 15 mm) were induced surgically in the back of young white Yorkshire pigs (10 to 15 kg) at a depth of about 1.0 mm by using a modified Castroviejo electrokeratome (Storz, St. Louis, MO; modified by Brownwells, Montezuma, IA).[11] The wound healing model described here is a modification of that reported by Alvarez et al.[23]

In Situ *Hybridization*

In situ hybridization was performed according to Hoefler et al.[24] Sections (2-mm thick) from fresh biopsy specimens were fixed in ice-cold 4% paraformaldehyde for 2 to 8 hours and allowed to sink in 30% sucrose/phosphate-buffered saline (PBS) overnight at 4 C to decrease freezing artifacts. The fixed tissues were then embedded in OCT (Miles Laboratories, Inc., Naperville, IL) for cryostat serial sectioning (8 μ). The sectioned tissues were rehydrated in PBS at room temperature and immersed in 0.1 mol/L glycine/PBS for 5 minutes, followed by a 15-minute immersion in 0.3% Triton X-100/PBS. The tissues were washed in PBS and then incubated in 1 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO), 0.1 mol/L Tris, pH 8.0, and 0.05 mol/L ethylenediaminetetraacetic acid, pH 8.0, at 37 C for 30 minutes to improve penetration of the hybridization probes. To stop proteolysis and

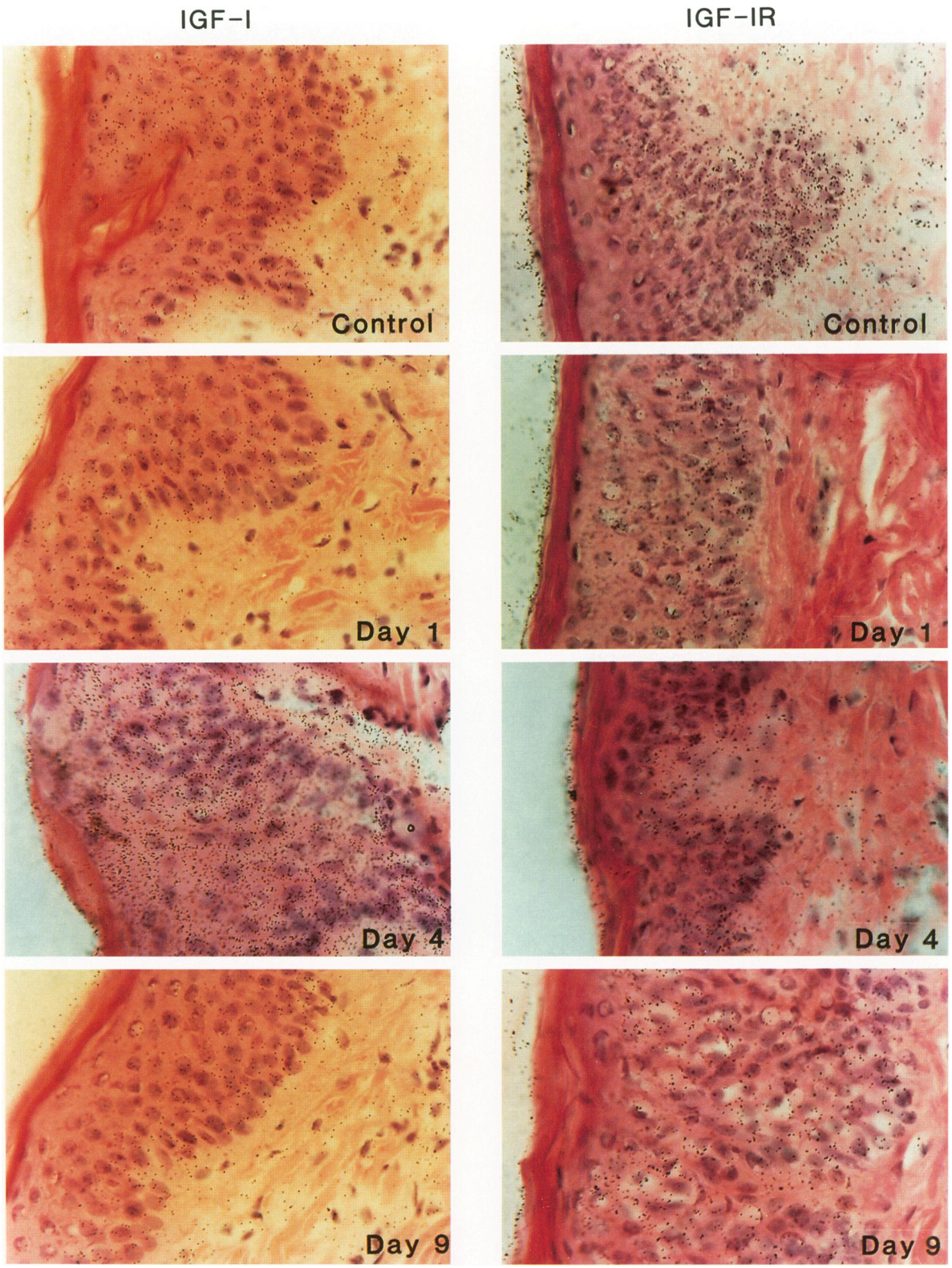

Figure 4. *Expression of IGF-I and IGF-I R mRNAs in skin epithelial cells before and after acute cutaneous injury. Notice that IGF-I and IGF-I R mRNAs are normally expressed in the epithelial cells of control, uninjured tissues. IGF-I mRNA expression can be seen in all biopsy specimens after injury with a substantial increase at day 4. The expression of IGF-I R mRNA remained strong before and after injury. Original magnification:* ×630.

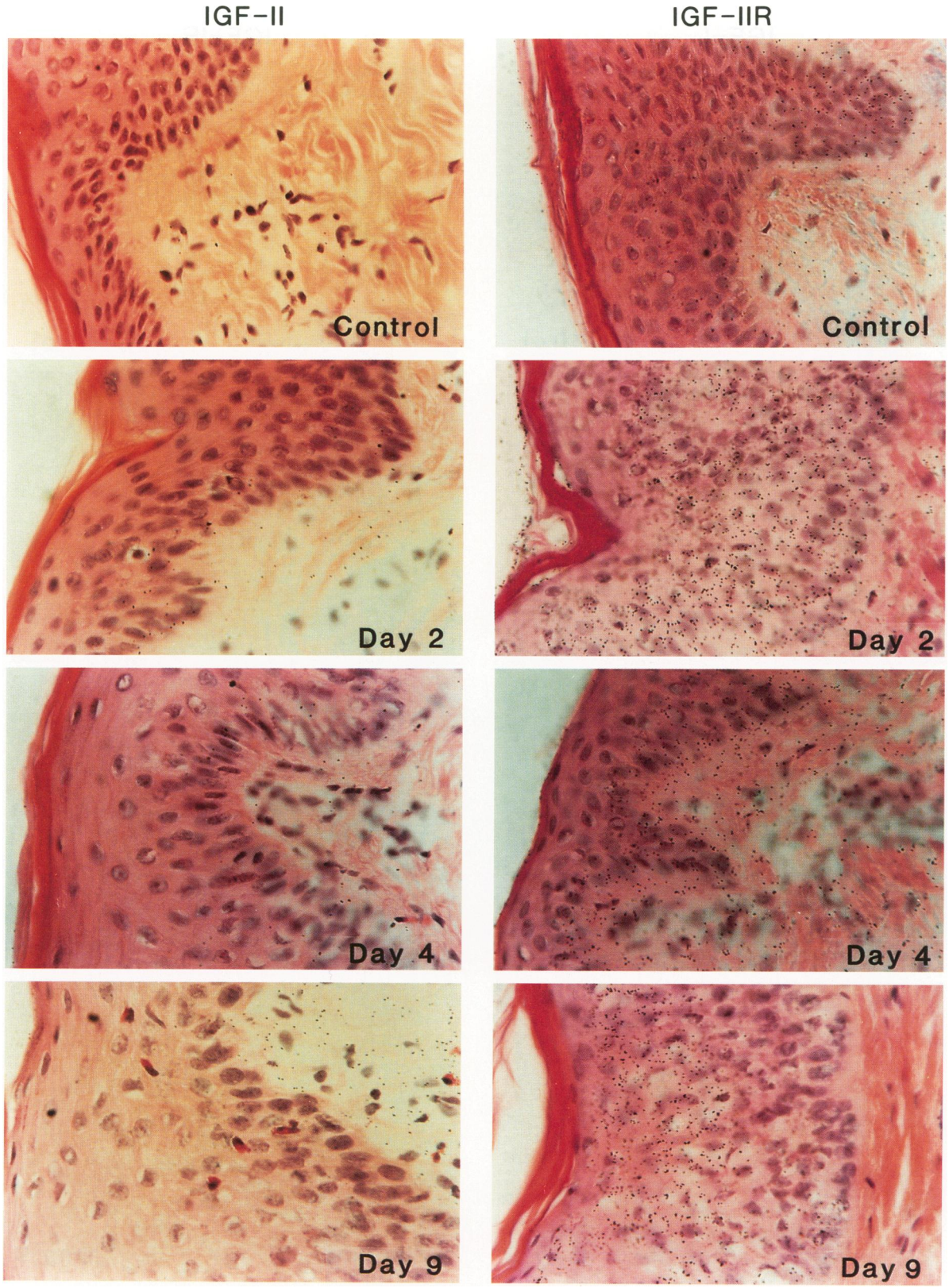

Figure 5. *Expression of IGF-II and IGF-II R mRNAs in skin epithelial cells before and after cutaneous injury. There was no significant expression of IGF-II mRNA in the epithelial cells before or after acute cutaneous injury. IGF-II R mRNA was normally expressed in the epithelium of control, uninjured tissue, and the expression was maintained after injury. Original magnification: ×630.*

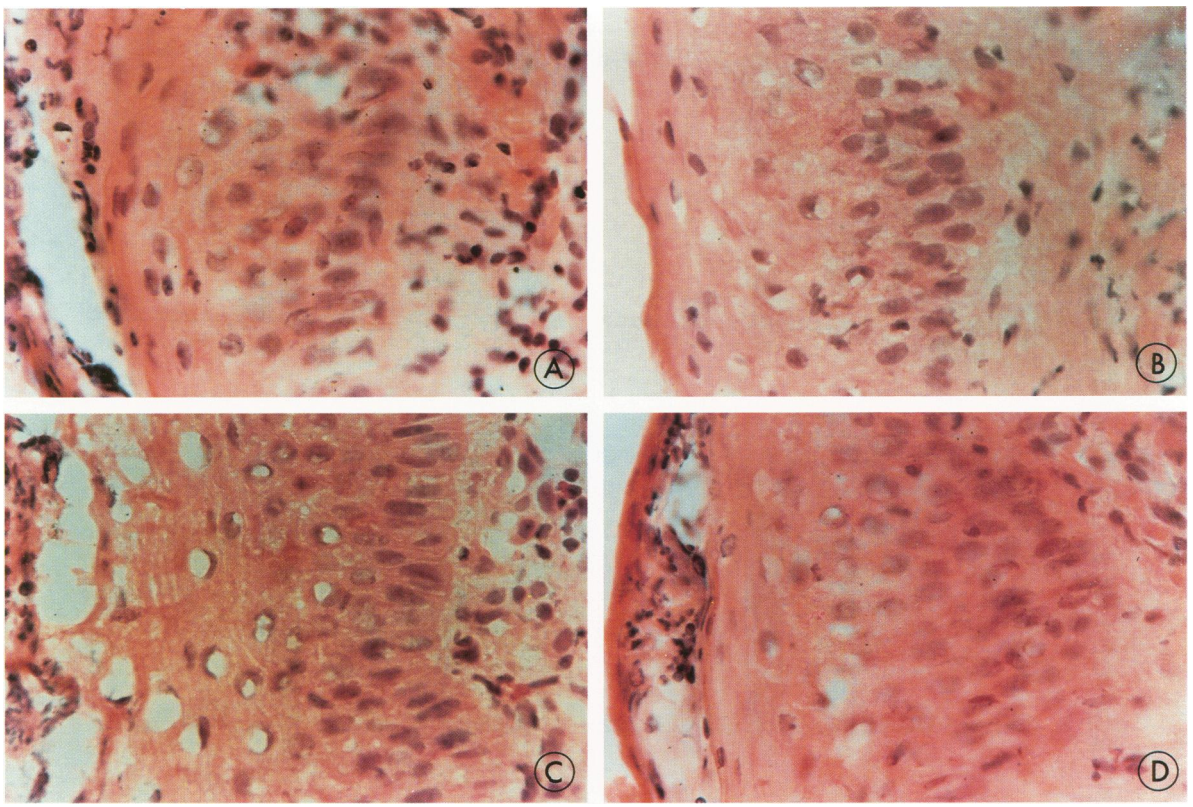

Figure 6. *This figure presents control data derived from* in situ *hybridization of skin biopsy specimens using noncomplementary sense riboprobes. Day 1 skin biopsies were hybridized with TGF-α* **(A)**, *EGF-R* **(B)**, *and bFGF* **(D)** *sense riboprobes. Day 5 biopsy specimen was hybridized with aFGF* **(C)** *sense riboprobe. In all cases there was no significant hybridization. In contrast, the corresponding specimen hybridized with complementary anti-sense riboprobes demonstrated significant hybridization* **(Figures 1** *and* **2)**. *Original magnification: ×630.*

decrease loss of mRNA, the tissue sections were treated with 4% paraformaldehyde/PBS. The sections were incubated in freshly prepared 0.25% acetic anhydride in triethanolamine, 0.1 mol/L, pH 8.0, for 10 minutes to reduce background in autoradiographs and then prehybridized in 50% formamide/2× standard saline citrate at 42 C for at least 15 minutes. After that, the tissue sections were drained and received 10 µl of hybridization mixture, containing hybridization buffer (50% formamide, 2× standard saline citrate, 10% dextran sulfate, 0.25% bovine serum albumin, 0.25% Ficoll 400, 0.25% polyvinyl-pyrolidine 360, 0.5% sodium dodecyl sulfate, and 250 µg/ml denatured salmon sperm DNA) and the ^{35}S-labeled anti-sense RNA probe, 3×10^5 counts per minute. The sections were incubated in a moist chamber at 42 C for 16 hours, followed by treatment with standard saline citrate, RNAse digestion (20 µg/ml for 30 minutes), and dehydration in graded alcohol solutions containing 0.3 mol/L ammonium acetate. Autoradiography of triplicate sections from each tissue was performed using NTB2 (Eastman Kodak) and were developed and stained with hematoxylin and eosin at weekly intervals for over a period of 3 weeks. Data presented here are from a 2-week exposure. The specificity of the *in situ* hybridization was controlled by parallel hybridization of serial tissue sections with control, noncomplementary (sense) riboprobes. The anti-sense RNA probes used in these studies were for TGF-α,[25] EGF-R,[26] aFGF,[27] bFGF,[28] IGF-I,[29] type I IGF-I receptor,[30] IGF-II,[31] and type II IGF-II receptor.[32]

Complementary (c)RNA probes were prepared as described in Hoefler et al,[24] using the Promega Gemini II system. To test the integrity of the probes 1 µl (2×10^6 cpm) of the probe was run on a 5.5% formaldehyde-reducing gel, transferred onto a nytran membrane, and washed in water. The membrane was then exposed to an x-ray film for 1 to 3 hours. The presence of a single transcript indicated that the probe was intact.

Results

Expression of TGF-α and EGF-R mRNAs

As shown in Figure 1, there is no significant expression of TGF-α mRNA and its receptor, the EGF-R,

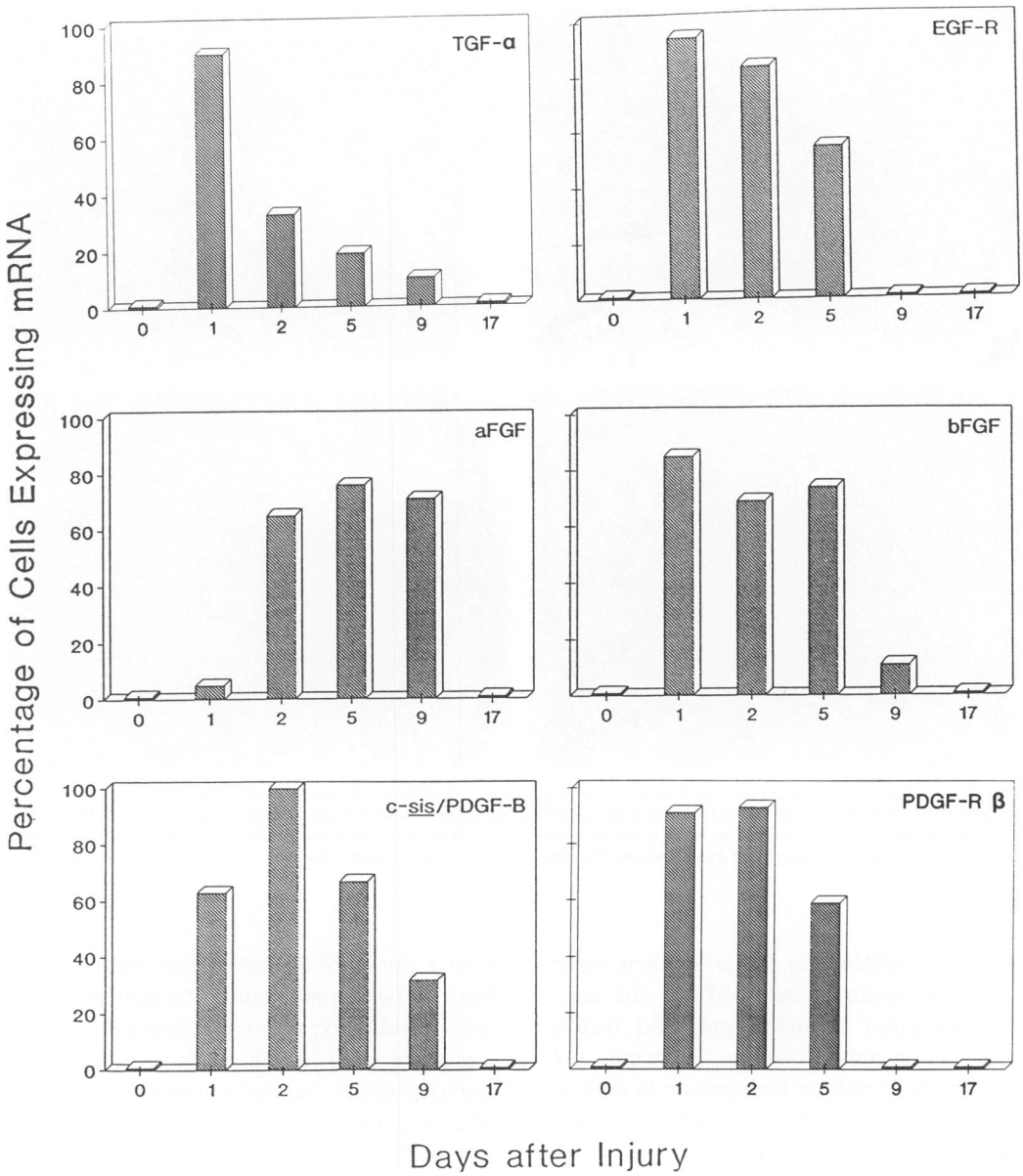

Figure 7. *Quantitation of TGF-α, EGF-R, aFGF, bFGF, c-sis/PDGF-2(B), and PDGF-R β mRNA expression in skin epithelial cells before and after acute cutaneous injury. Notice the lack of expression in the epithelial cells of control, uninjured tissue.*

mRNA in the epithelial cells of control, uninjured tissues (Figure 1). Injury induced the expression of both TGF-α and EGF-R mRNAs in the epithelial cells of the wounded area. Expression of TGF-α mRNA in the epithelial cells adjacent to the injured tissue can be seen within 1 day of injury; it declined by day 2 and was completely suppressed by day 9. EGF-R mRNA was expressed within 1 day of injury, declined gradually over 5 days, and was completely suppressed by day 9, which coincides with the time of complete reepithelialization of the wounded tissue. The EGF-R mRNA expression was localized primarily in the proliferating epithelial cells at the wounded site and in epithelial cells localized in immediate proximity of the wound (Figure 1).

Expression of aFGF and bFGF mRNAs

There was no significant expression of aFGF and bFGF mRNAs in the epithelial cells of control, uninjured tissue (Figure 2). Injury induced the expression of aFGF mRNA in the epithelial cells at the wound site, and this expression was apparent from day 2 to day 9 after injury. A strong expression at

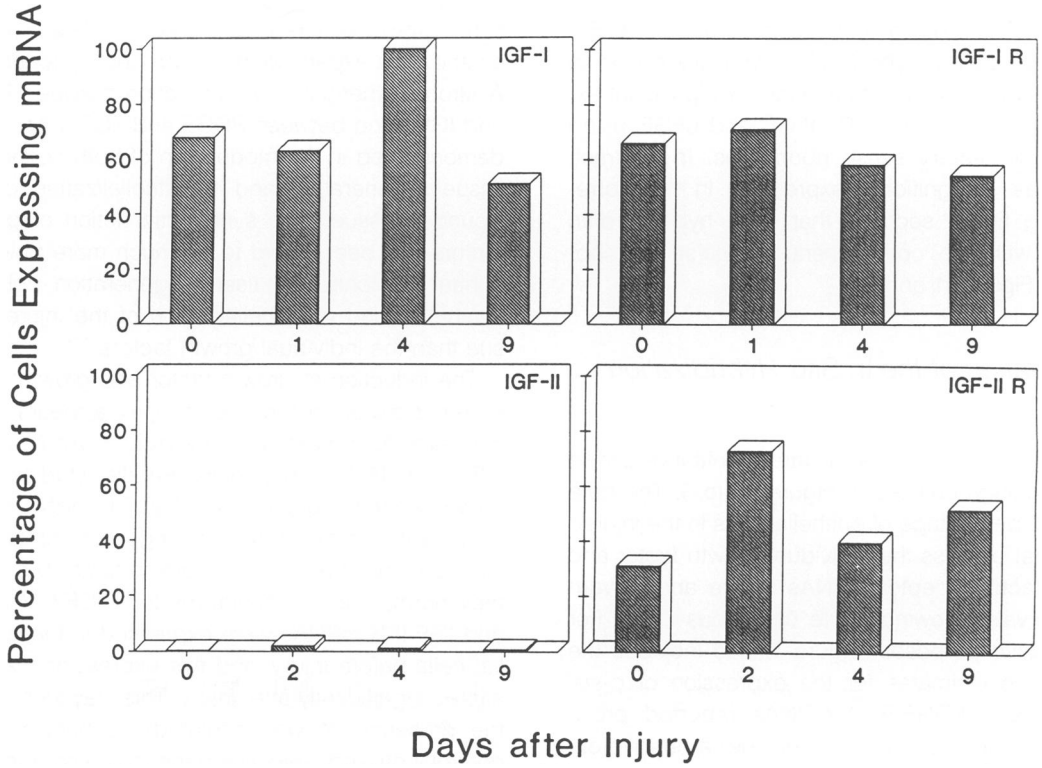

Figure 8. *Quantitation of IGF and IGF-receptor mRNA expression in skin epithelial cells before and after acute cutaneous injury. There is no significant expression of IGF-II mRNA before or after injury. IGF-I, IGF-I R, and IGF-II R mRNAs were expressed in the epithelial cells of both control, uninjured tissues and in tissue specimens obtained after injury.*

day 9 can be seen primarily in the basal layers of the proliferating epithelium. The expression of aFGF mRNA was completely suppressed by day 17. A strong expression of bFGF mRNA in the epithelial cells was seen within one day of injury; it remained strong for 5 days, declined by day 9, and was totally suppressed by day 17 (Figure 2). It seems that there is a differential expression between aFGF and bFGF mRNAs in response to injury, in that the aFGF expression increased progressively over a 5-day period and was still strong by day 9, whereas the strongest expression of bFGF mRNA was seen in day 1, declining progressively to near background levels by day 9. The expression after injury seen in Figures 1 and 2 was confined in the epithelium adjacent to the injured region and in the proliferating epithelium at the site of injury. There was no significant expression in the distal intact skin epithelium as shown in the example presented in Figure 3.

Expression of IGF-I and IGF-I Receptor (IGF-I R) mRNAs

As described above, intact, uninjured skin epithelial cells did not express TGF-α, EGF-R, aFGF, or bFGF mRNAs. In contrast, both IGF-I, and its receptor, IGF-I R, mRNA are normally expressed in the control, uninjured epithelial cells (Figure 4). Following injury, the expression levels of IGF-I mRNA remained unchanged with a significant increase seen at about day 4 after injury. The expression of IGF-I R mRNA remained largely unchanged during and after the healing process (Figure 4).

Expression of IGF-II and IGF-II Receptor (IGF-II R) mRNAs

There was no detectable expression of IGF-II mRNA in the skin epithelial cells before or after injury (Figure 5). In contrast, significant expression of IGF-II R mRNA can be seen in the epithelial cells both before and after injury (Figure 5). The absence of significant expression of IGF-II mRNA following injury may signify that induction of gene expression by injury is not a generalized event but may involve selective mechanisms for individual growth factors and/or their receptors.

The specificity of the *in situ* hybridization studies presented in Figures 1 to 5 was controlled by parallel hybridization using noncomplementary sense riboprobes. There was no significant expression for

TGF-α, EGF-R, aFGF, bFGF, IGF-I, IGF-II, IGF-I R, or IGF-II R mRNAs using control sense riboprobes. Examples of control hybridization data are shown in Figure 6, which demonstrate a lack of significant expression of TGF-α, EGF-R, aFGF, and bFGF, using noncomplementary sense riboprobes. In contrast, there was a significant expression in the corresponding tissue sections that were hybridized in parallel with the complementary anti-sense riboprobes (Figures 1 and 2).

Quantitation of the In Situ *Hybridization Data*

Figures 7 and 8 summarize the quantitative profile of the findings outlined in Figures 1 to 5. The data show the percentage of epithelial cells in the injured tissue that express the individual growth factor and growth factor receptor mRNAs before and at various intervals following acute cutaneous injury. For comparative purposes, Figure 7 also includes the quantitative estimates for the expression of c-*sis*/PDGF-2 and PDGF-R β mRNAs reported previously.[10] In these studies, we consider a positive expression to be the appearance of more than three grains over the epithelial cells.

Discussion

The major findings in the present studies can be summarized as follows: a) acute injury can induce in the skin epithelial cells of the wounded tissue the expression of genes encoding for potent growth factors and growth factor receptors; b) upon completion of the healing process, the expression of the genes induced by injury is totally suppressed in the epithelial cells of the healed tissue. Thus, growth factor gene expression and suppression in the course of normal wound healing reflects the presence of well-controlled molecular mechanisms that serve for regulated tissue regeneration and remodeling. For example, the expression of the PDGF-2(B) homodimer and its receptor in both epithelial cells and connective tissue fibroblasts may contribute to the autocrine proliferation of epithelial cell leading to reepithelialization, to connective tissue cell migration and proliferation, and to stimulations of matrix synthesis by the fibroblasts, including collagen synthesis.[11] TGF-α,[33–36] aFGF,[27,37,38] and bFGF[39–42] are mitogenic for fibroblasts, as well as for endothelial and epithelial cells. These potent mitogens may contribute to connective tissue regeneration, neovascularization, and reepithelialization of the injured tissue. In addition to their individual actions, growth factors may act synergistically *in vivo* as shown in experimental wound healing studies.[4,8] A strong synergistic *in vivo* action between PDGF and IGF-I and between PDGF and TGF-α has been demonstrated in the modulation of both connective tissue regeneration and reepithelialization of the wounded tissue. This synergistic action of growth factors has been found to be much more potent in enhancing connective tissue regeneration, collagen synthesis, and reepithelialization of the injured tissue than the individual growth factors.[4,8]

The induction of growth factor and growth factor receptor genes at the site of injury appears to be selective, as indicated by the lack of expression of IGF-II mRNA following injury. Parallel studies have shown a strong expression of IGF-II mRNA in the malignant astrocytes and meninges of primary human astrocytoma and meningioma tumor but not in their normal cellular counterparts.[43] IGF-I, IGF-I R, and IGF-II R mRNAs were expressed in the epithelial cells before injury, and this expression was not altered significantly after injury. This may also reflect the presence of well-regulated mechanisms that control localized, selective gene induction and suppression in response to acute injury. The nature of the mechanisms involved in the induction of growth factor gene expression by acute injury and gene suppression by healing are not known. The present studies provide an *in vivo* model for the investigation of these physiological molecular mechanisms that regulate tissue repair and remodeling in response to injury.

In contrast to acute injury, chronic injury seems to induce *in vivo* growth factor and receptor gene expression that is not suppressed. For example, epithelial cells in lung biopsy specimens of patients with idiopathic pulmonary fibrosis express high levels of c-*sis*/PDGF-2(B) mRNA.[44] Malignant epithelial cells in primary human lung cancer specimens were shown to express both c-*sis*/PDGF-2(B) and PDGF-R β mRNAs.[45] This expression is similar to that seen in normal skin epithelial cells following acute cutaneous injury, except the in acute injury the expression is suppressed upon healing, whereas the expression in idiopathic pulmonary fibrosis and in lung cancer tumor cells is nonsuppressible. It seems that chronic injury, in contrast to acute injury, causes a malfunction of the mechanisms that normally regulate gene suppression.[46]

In the present study, we report the expression of growth factor and growth factor receptor mRNAs in the epithelial cells of the injured tissue. Preliminary data demonstrate that this mRNA expression is ac-

companied by the expression of the corresponding protein products, as judged by *in situ* immunostaining using specific antisera. A detailed report on the protein expression and the mRNA expression seen in connective tissue fibroblasts and in inflammatory cells will appear elsewhere.

Acknowledgments

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