GM-CSF Activates Regenerative Epidermal Growth and Stimulates Keratinocyte Proliferation in Human Skin In Vivo

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Granulocyte/macrophage-colony-stimulating factor (GM-CSF), an immunomodulator of hematopoietic cells, has also been shown to stimulate human keratinocyte proliferation in vitro and speed healing of wounds in the skin of lepromatous leprosy patients. In this study we have examined the in vivo effects of recombinant human GM-CSF on epidermal keratinocyte proliferation and on expression of proteins marking regenerative epidermal growth. Skin biopsies from GM-CSF injected cutaneous sites were obtained between 1 and 6 d following administration of 7.5 or 15 μg of the growth factor. Activation of keratinocyte proliferation, quantified as the expression of the Ki67+ nuclear antigen, was noted 1 d following GM-CSF administration. A regenerative epidermal phenotype, demonstrated by immunohistochemical staining of cellular proteins involucrin, filaggrin, and keratin 16, was similarly noted as early as 1 d following GM-CSF injection. This phenotype persisted as late as 6 d postinjection. These results suggest that GM-CSF injection into human skin induces keratinocyte proliferation as well as regenerative differentiation of the epidermis. To date no other cytokine has been shown to be mitogenic for human keratinocytes both in vivo and in vitro or to alter keratinocyte differentiation along the "alternative" or regenerative pathway. J Invest Dermatol 103:601-604, 1994

Materials and Methods

Patients Twenty patients with lepromatous leprosy were selected for GM-CSF administration as previously described [7]. Recombinant GM-CSF was supplied by Sandoz AG (Basel, Switzerland). Intradermal injections of either 7.5 μg or 15.0 μg of GM-CSF produced in CHO cells (GM 89-107) (batch 4026 0188) were administered daily as described [7]. Patients were randomly assigned to receive a repeated injection of GM-CSF at a previously administered site. A 4-mm punch biopsy was taken on entry to the study. Injection site biopsies were performed at specific time intervals after GM-CSF administration. All 20 patients were biopsied at time 0. Four patients were biopsied after 24 h, five after 48 h, four after 72 and 96 h, and three after 120-144 h. Ten additional biopsies were performed on patients who underwent re-injection. A final biopsy was taken from an uninvolved site on day 11. All injections were given in skin of the back of patients [7].

Immunohistochemistry Biopsy specimens were fixed in neutral buffered 10% formalin overnight, embedded in paraffin, and sectioned. Sections were deparaffinized in xylene and decreasing concentrations of ethanol mixed with phosphate-buffered saline solution (PBS). Two distinct immuno-
noperoxidase techniques were initially performed. The specimens to be stained with the Ki67 nuclear antibody (AMAC, Inc., Westbrook, Maine) underwent trypsinization for 10 min and were fixed in cold, 100% acetone. Activation of cellular proliferation was quantified by the counting of keratinocytes expressing Ki67+ nuclei located in the basal or immediately suprabasal epidermis and presented as a percentage of 100 total basal cells. Specimens to be examined for keratinocyte cytoplasmic proteins were fixed with acetone:methanol, 1:1. The antibodies directed against filaggrin and involucrin [8] were obtained from Biomedical Technology, Inc., MA. Monoclonal antibody AE-1 was a generous donation from Dr. T. T. Sun. The regenerative maturation phenotype was defined as premature expression of filaggrin and involucrin by lower spinous keratinocytes and induced expression of keratin 16 (detected by antibody AE-1). Immunoperoxidase studies were performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as described previously [12,13]. Negative controls were performed for all immunoperoxidase studies.

**Determination of Epidermal Thickness** Epidermal thickness was evaluated by direct examination of hematoxylin-stained sections using a computer-based image digitizing system (Southern Micro Instruments, Inc., Atlanta, GA) [7].

**Statistical Analysis** Biopsy specimens were divided into pre- and post-treatment groups. Differences in the incidence of Ki67+ cells and the expression of a regenerative maturation phenotype were analyzed using a chi-square model.

**RESULTS**

Injection of GM-CSF intradermally into the skin of patients with lepromatous leprosy produced epidermal thickening (acanthosis) that peaked by 24-48 h, persisted for up to 6 d after injection, and had returned fully to basal levels by 11 d (Fig 1A). To establish whether acanthosis was the result of keratinocyte hyperplasia, hypertrophy, or a combination of the two, skin section sites were stained with antibodies detecting the nuclear antigen Ki67 (hyperplasia) and the cellular proteins filaggrin, involucrin, and keratin 16. Intradermal injection of human rGM-CSF increased keratinocyte proliferation as demonstrated by an enhanced number of Ki67+ keratinocytes in the epidermis. The number of Ki67+ keratinocytes was enhanced by 24 h and peaked within 48 h after rGM-CSF injection (Fig 1B). There were still substantial increases in the number of Ki67+ nuclei after 96 h.

Because the peak of epidermal thickening preceded peak keratinocyte proliferation, we examined the role of keratinocyte differentiation in GM-CSF–induced epidermal acanthosis. The ability of rGM-CSF injection to induce regenerative maturation was assessed by staining with antibodies directed against filaggrin, a protein normally associated with keratin filaments in keratohyaline granules, and involucrin, a membrane protein crosslinked by transglutaminase as part of normal keratinocyte terminal differentiation. Although these cellular proteins are normally expressed in the granular layer of the epidermis, premature expression of filaggrin and involucrin occurs in lower spinous keratinocytes in regenerating epidermis. Increased premature expression of filaggrin was observed as early as 1 d after rGM-CSF injection (Fig 1C). Similarly, the incidence of premature expression of involucrin in lower spinous keratinocytes was also noted as early as 1 d after the administration of rGM-CSF (Fig 1C). The increased presence of confluent regenerative maturation was detected for both involucrin (p = 0.009) and filaggrin (p = 0.067) following GM-CSF administration. The expression of AE-1 reactive keratins in intrafollicular keratinocytes was absent prior to GM-CSF injection; however, several patients demonstrated focal induced expression of the keratin-16 protein following cytokine administration (see below).

Figure 2 illustrates the histologic staining patterns of Ki67+ keratinocyte nuclei, involucrin, filaggrin, and keratin 16 in skin following GM-CSF injection. A single injection of rGM-CSF resulted in keratinocyte activation in vivo as evidenced by increased proliferation and increased expression of the regenerative maturation phenotype. This growth-activated phenotype is identical to that observed in acute wounds and hyperproliferative epidermal diseases [14].

**Figure 1. Effects of GM-CSF on human epidermis in vivo.** A) Epidermal thickness in control skin (day 0) or following intradermal GM-CSF injection (days 1–4), expressed as average thickness in microns. B) Keratinocyte proliferation in control skin (day 0) or following GM-CSF injection (days 1–4), as determined from the percentage of basal keratinocytes expressing the Ki67 nuclear protein. C) Expression of involucrin (solid bars) or filaggrin (hatched bars) in a pattern of regenerative epidermal growth/maturation as assessed in control skin (day 0) or following GM-CSF injection (days 1–4).
Figure 2. GM-CSF alters expression of proliferation and differentiation-associated proteins in human epidermis after in vivo administration. Histologic sections showing histochemical detection of the Ki67 protein (A,B); involucrin (C,D); filaggrin (E,F); or keratin 16 (G,H) in control (pre-injection) skin (left column: A,C,E,G) or in sites following intradermal GM-CSF injection (right column: B,D,F,H). Involucrin staining in D is shown 1 d after GM-CSF administration, whereas Ki67 (B), filaggrin (F), and keratin 16 (H) are shown 3 d following GM-CSF administration (arrows, positive staining reactions in GM-CSF injected sites). Faint-to-moderate pigmentation of basal keratinocyte cytoplasm is due to melanization in all micrographs shown. All micrographs are shown at the same magnification; bar in A, 200 μm.
Because GM-CSF is produced not only by immune cells but also by keratinocytes [15], it is a potential autocrine or paracrine regulator of epidermal growth. Under normal conditions, keratinocyte GM-CSF gene expression and production of GM-CSF are limited. However, under several conditions, including infection and tissue injury, the local production of interleukin 1 and tumor necrosis factor-α lead to increased expression of GM-CSF mRNA and increased production of GM-CSF [16,17]. It is postulated that an increase in GM-CSF would result in increased wound healing, as well as activation of macrophages and Langerhans cells, thereby enhancing the host's local cellular immune defense. A common occurrence with injection [19]. Histopathologic characterization of the widespread edema, occasional keratinocyte dysplasia, and variable lymphocyte infiltration [18]. These effects clearly indicate the route of action and differentiation. As acknowledged, however, that the effects of GM-CSF could be mediated indirectly through secondary cytokines in human skin [7].

Leukin-6 is a mitogen for human keratinocytes in culture and in vivo. Transforming growth factor-α production by keratinocytes in a receptor expression, suggesting that GM-CSF does not function as a pan-activator of autocrine growth circuits for epidermal keratinocytes.

Previous studies have shown that physical trauma to the epidermis, e.g., the removal of the stratum corneum by tape-stripping, can induce the regenerative epidermal phenotype, an alternative pathway of keratinocyte differentiation. Interestingly, this alternative pathway of keratinocyte differentiation is persistently present in uninjured epidermis associated with inflammatory skin pathologies such as psoriasis and lichen plans. Although keratinocyte proliferation is increased in many of these disorders and an increased abundance of several cytokines has been noted, no molecular inducers of regenerative epidermal growth have previously been identified. The results of the present study identify GM-CSF as a probable regulator of regenerative epidermal growth, although other cytokines might also induce this epidermal phenotype. The production of GM-CSF by numerous cellular elements associated with infiltrating cells in skin wounds, psoriasis, and other inflammatory dermatoses, suggests that GM-CSF could be the molecular link between cellular immune elements infiltrating skin and induction of an altered epidermal phenotype. GM-CSF may also have a potentially therapeutic role in modifying the healing of acute or chronic skin wounds.

REFERENCES