ORIGINAL ARTICLE

Keratinocyte Growth Stimulation by Granulocyte-macrophage Colony-stimulating Factor (GM-CSF)

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Abstract. Human granulocyte-macrophage colony stimulating factor (GM-CSF) is a 22 kD glycoprotein derived from activated T cells, endothelial cells, fibroblasts and macrophages. It stimulates the proliferation and differentiation of haematopoietic cells and certain nonhaematopoietic cells. This study investigated the effect of GM-CSF on cultured human keratinocytes and determined whether these cells express GM-CSF receptors. Measurement of keratinocyte growth by image analysis showed that GM-CSF mildly stimulated the growth of keratinocytes. With 4 ng/ml GM-CSF the growth of primary keratinocytes and subcultured keratinocytes was only stimulated up to 25% and 18% of the control cell level, respectively. Human keratinocytes were incubated with GM-CSF at 4 ng/ml for 4 days to induce receptor expression. These cells showed only a weak positive reaction on affinity labelling using digoxigenated GM-CSF. We conclude that keratinocyte growth may be stimulated by GM-CSF but that the presence of GM-CSF receptors on these cells is equivocal. (Keio J Med 46 (4): 184–187, December 1997)

Key words: GM-CSF, GM-CSF receptors, keratinocytes, cell culture, digoxigenin

Introduction

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 22 kD glycoprotein derived from activated T cells, endothelial cells, fibroblasts and macrophages. It is active on a variety of haematopoietic cells and nonhaematopoietic cells.¹ It is also produced by keratinocytes² and stimulates their growth *in vitro*.³ Hancock *et al* showed that IFN- γ and TGF- β inhibited keratinocyte growth, whereas IL-3 and GM-CSF stimulated keratinocyte growth in an *in vitro* assay.³

GM-CSF receptors have been demonstrated in non-haematopoietic cells such as small cell lung cancer cell lines,⁴ colon adenocarcinoma cell lines,⁵ melanoma cell lines,⁶ breast cancer cell lines,⁷ primary human melanoma cells,⁶ normal human endothelial cells⁸ and normal human placenta and a trophoblast cell line.⁹ However, epithelial cells from normal oral mucosa were negative for this receptor.¹⁰

The aims of this study were to assess the effects of GM-CSF on (a) keratinocyte growth and (b) induction

of GM-CSF receptor expression on human keratinocytes *in vitro* using affinity labelling.

Materials and Methods

Cell culture

Normal human epidermal keratinocytes were initiated and subcultured using our modification¹¹ of Rheinwald and Green's technique.¹² Epidermal cells were maintained in keratinocyte growth medium (KGM), a mixture of Dulbecco's modification of Eagle's medium (DMEM) (Gibco, Life Technologies Ltd, Paisley, UK) and Ham's F12 (Gibco) (3:1) containing 5% foetal calf serum (Gibco), hydrocortisone (Sigma) (0.4 mg/ml), cholera toxin (Sigma) (10 ng/ml), insulin (Sigma) (5 mg/ml), transferrin (Sigma) (5 mg/ml), triiodo-thyronine (Sigma) (2×10^{-11} M) and epidermal growth factor (Gibco) (10 ng/ml). During the first 7 days of primary culture the medium also contained penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco). Our basal

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medium was serum free medium (SFM) (Gibco) and contained growth-limiting concentrations of bovine pituitary extract (BPE) (Gibco) (2.5 mg/ml). At 80–90% confluence, the 3T3 cells (IGN Flow) were removed with 0.02% EDTA (BDH Chemicals Ltd, Poole, UK) and the keratinocytes were trypsinized (0.25% trypsin (Gibco)/0.02% EDTA) to single cell suspensions, washed in KGM, and seeded in 24-well plates (Nunc, Intermed.) for growth assays. 3T3 fibroblasts were maintained in DMEM containing 10% new-born calf serum (Gibco).

Keratinocyte growth assays

The effects of GM-CSF on keratinocyte growth were assessed *in vitro* by measuring the percentage of the well surface covered by keratinocytes using the Optomax V image analysis system. Primary or subcultured normal human keratinocytes were seeded in 24-well plates at 5×10^3 cells per well. At 48 h of culture, the medium was replaced with SFM containing growth limiting concentrations of BPE (2.5 mg/ml) supplemented with or without recombinant human (rh) GM-CSF (kindly provided by Sandoz Pharmaceuticals Ltd, Sulley, UK) and the cells were cultured for 4 days. Image analysis was performed on triplicate wells to measure the area of keratinocyte growth after staining with 0.1% crystal violet.

Labelling of GM-CSF with digoxigenins

A reconstituted solution of unglycosylated rh-GM-CSF (200 mg/ml) (Sandoz) was diluted in 500 ml of Dulbecco's salt solution (pH = 7.2) (DSS) containing 1.5 mg digoxigenin-3-O-succinyl-2-(N-maleimido)-ethylamide (compound A) and 5 mg digoxigenin-3-O-methylcarbonyl-aminocaproic acid-N-hydroxysuccinimide ester (compound B) (Boehringer Mannheim) at a final dilution of 100 ng/ml and incubated for 2 hours at room temperature as described by Roncaroli *et al.*¹⁰ A solution of 500 ml of DSS containing compound A and B without GM-CSF and 500 ml of the same buffer were used as control solutions. Digoxigenated GM-CSF was mixed with 5 mg/ml of unlabelled factor and used as a blocking solution to test the specificity of the reaction.

Detection of GM-CSF receptors on cultured keratinocytes

Expression of the GM-CSF receptor on keratinocytes was studied by affinity labelling using digoxigenated-GM-CSF as described previously.¹⁰ Primary epidermal keratinocytes were seeded onto coverslips for affinity labelling and grown in KGM. At 50% confluence, after activation of keratinocytes by culture with 4 ng/ml

GM-CSF for 4 days, cells were washed in phosphate-buffered saline (PBS) and cultured in basal SFM in the absence of GM-CSF for 24 h to induce surface expression of the receptor. The cells were then washed with DSS and fixed in cold absolute methanol (-24°C) for 10 min and incubated with one of the following, after inhibition of endogenous peroxidase with 3% H_2O_2 for 20 min; (1) digoxigenated GM-CSF; (2) blocking solution; (3) digoxigenated compounds at the labelling concentration without GM-CSF; (4) DSS. All samples were incubated for 2 h at room temperature, post-fixed with 4% paraformaldehyde for 10 min and then incubated with anti-digoxigenin polyclonal antibody (Boehringer Mannheim) at final dilution of 1/100 for 2 h after washing in DSS. A peroxidase-conjugated donkey anti-sheep Ig secondary antibody (Sigma) was employed for 30 min at a dilution of 1/1000. The reaction was developed in 3,3'-diaminobenzidine for 30 min. The specimens were dehydrated in progressive alcohols, cleared in xylene and mounted in synthetic medium.

Results

GM-CSF had a mild stimulatory effect on primary keratinocytes at all concentrations between 4 to 16 ng/ml ($p < 0.05$) (Fig 1). A flattened dose response curve was seen with peak stimulation occurring at 4 ng/ml in three independent experiments with primary cells and

Fig 1 The effect of GM-CSF on keratinocyte growth. Primary and passaged keratinocytes were seeded in 24-well plates and cultured for 6 days. GM-CSF was given twice on days 2 and 4. Image analysis was performed and the area of cell growth measured. Results are expressed as the mean $\pm$ SEM of three independent experiments for primary cells and as the mean of three wells for passaged cells. A flattened dose response curve was seen with peak stimulation occurring at 4 ng/ml. *Significantly different from controls, $p < 0.05$.

in an experiment with passaged cells. Primary keratinocytes were only stimulated to grow up to 25% more than controls, while subcultured keratinocytes were not significantly stimulated.

Affinity labelling of GM-CSF receptors showed weak positive staining (Fig 2 A). Control tests with blocking solution (Fig 2 B), digoxigenin labelling solution (Fig 2 C) and phosphate buffered saline (Fig 2 D) were negative.

Discussion

Healing of cutaneous wounds follows a sequence of cellular events. Inflammation, angiogenesis, fibroplasia, wound contraction and epithelialisation result in the

final formation of a scar. Vascular, inflammatory and fibroblastic cells release cytokines locally at wound sites. GM-CSF can be produced by many of the cell types which are present within granulation tissue. It is therefore possible that GM-CSF might play an important role in wound healing and that application of GM-CSF could have beneficial effects in situations where healing is delayed such as recalcitrant ulcers.

The possibility that GM-CSF might possess stimulatory activity on keratinocyte growth has been examined using cultured normal human keratinocytes. Although GM-CSF slightly stimulated the growth of keratinocytes in agreement with other assays, the effect of the factor was much smaller in this system than as described previously³ and appeared to be reduced in subcultured cells. In our experiments, peak stimulation with GM-CSF occurred at 4 ng/ml, which was at a higher concentration than that used by Hancock *et al*,³ in which the peak stimulation occurred at 0.6 ng/ml. This difference in effective GM-CSF concentration might be due to differences in the culture system or to the timing of GM-CSF addition.

Kaplan *et al*¹³ showed that intradermal injection of GM-CSF in patients with leprosy induced epidermal thickening, Langerhans cell recruitment in the dermis and enhanced wound healing. They stated that there was a possibility that they might be inducing the synthesis and release of keratinocyte GM-CSF or other molecules that extended the response, and said that more accurate evaluation should be carried out in normal skin in the future. Our results also encourage us to pursue the possible action of this cytokine in wound healing in chronic non-healing ulcers in man. However, other cytokines may also play important roles in this process.

The expression of GM-CSF receptors by both haematopoietic and non-haematopoietic cells has been a subject of recent interest. However, the lack of a specific anti-GM-CSF receptor antibody has meant that detection of GM-CSF receptor expression is usually accomplished using iodinated GM-CSF which often results in poor morphological evaluation. We employed a novel immunoenzyme technique for the detection of GM-CSF receptors, which has only been recently devised.¹⁰

GM-CSF receptors are classified as either high affinity or low affinity type.¹⁴ The biological actions of GM-CSF are mediated by binding to a specific high affinity receptor,⁴ and the function of the low affinity receptor is unknown. In the experiments described here, the reactivity on affinity labelling was very weak and scattered, and the distribution of the staining varied depending on the cells. This result suggests that the number of receptors is small and that expression of the receptor might depend on the cell culture conditions.

Fig 2 Affinity labelling of keratinocytes using digoxigenated GM-CSF. Human epidermal keratinocytes cultured on coverslips were activated by incubation with 4 ng/ml GM-CSF for 4 days and tested for GM-CSF receptor expression. A: Incubation with digoxigenin-labelled rh-GM-CSF. Cells showed a weak positive reaction. B: Incubation with digoxigenated-factor in the presence of excess of unlabelled GM-CSF to provide competition for the binding site. C: Incubation with digoxigenin without rh-GM-CSF. D: Incubation with phosphate buffered saline. Control cells (B, C, D) were all negative.

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