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AUTOCRINE MOTILITY FACTOR IS A GROWTH FACTOR*

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SUMMARY: Autocrine motility factor (AMF) is a 55 kDa cytokine which is produced and secreted by cancer cells and which regulates cell motility via binding to its receptor, a 78 kDa cell surface glycoprotein (gp78-AMFR), and activating a pertussis toxin (PT)-sensitive G-protein. AMF purified from HT-1080 human fibrosarcoma cells stimulates the growth and motility of 3T3-A31-fibroblasts at a concentration of 0.1 ng/ml or less. The expression of total as well as cell surface gp78-AMFR is down-regulated in contact-inhibited A31-fibroblasts and AMF stimulates the healing of experimentally wounded, density-arrested A31 monolayer cultures. This is the first report of the paracrine and mitogenic actions of AMF and the results presented here show that AMF functions as a growth factor and suggest a possible role for its activity in normal tissue regeneration and tumor cell dissemination.

Cell motility in response to extracellular signals is a prerequisite for a diverse range of normal and pathological processes, including embryonic tissue remodeling, inflammation, angiogenesis, invasion and metastasis (1-3). A group of factors has been described whose primary function is thought to be regulation of cellular kinesis. This includes autotaxin (4), migration stimulating factor (5,6) and hepatocyte growth factor (HGF), a potent mitogen for primary hepatocytes (7) and keratinocytes (8) which was shown to promote the scattering and invasiveness of epithelial tumor cells and was therefore co-defined as scatter factor (SF) (9-11). Host serum proteins and extracellular matrix breakdown products can influence cell motility as well (12-15). A tumor cell-derived autocrine motility factor (AMF) was identified by its ability to induce the directed and random migration of the self-producing cells (16). AMF stimulates cell motility via a receptor-mediated signaling pathway (17-18) and signal

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Abbreviations used are: AMF, autocrine motility factor; AMFR, receptor for autocrine motility factor; FITC, fluorescein isothiocyanate; gp78, 78 kDa cell surface glycoprotein; kDa, kilodalton; PBS, phosphate-buffered saline; PT, pertussis toxin.

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transduction following binding of AMF to its receptor is mediated by a PT-sensitive G-protein (17-19) and inositol phosphate production (20).

A wide range of growth factors which regulate target cell proliferation can exhibit multifunctional activity, affecting processes including protein secretion, receptor density, cellular migration and differentiation (21). Various growth factors act concurrently on cell proliferation and motility (22), including platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGF's), transforming growth factors (TGF's), insulin-like growth factor-I, tumor necrosis factor-alpha, interleukin-6, interferon-gamma, granulocyte and granulocyte-macrophage colony stimulating factors and bombesin (23), however, it has been demonstrated in another report that migration and proliferation of a murine large cell lymphoma are independently regulated by distinct growth and motility factors (24). In addition, EGF, FGF, and insulin stimulate DNA synthesis but have no effect on motility of human dermal fibroblasts (25) and HGF-SF can regulate motility without affecting the proliferation of certain epithelial cells (9-11). This implies that growth and migration may be controlled by separable regulatory pathways; however, since growth factors can influence both cell growth and motility, it suggests that they may be regulated in a cooperative manner by a single molecule. This made us question whether AMF acts as a growth factor or if its activity is only linked to motility. Interestingly, the receptor for HGF-SF was recently identified as the c-*met* proto-oncogene (26). Like gp78, c-*met* is a transmembrane glycoprotein which contains a kinase domain and intracellular phosphorylation sites which are thought to be involved in its activity and signal transduction mechanism. Both receptors are not only involved in stimulation of cell motility but also in mitogenic responses as well, therefore these receptors may share similar signaling pathways.

MATERIALS AND METHODS

Cells and Culture Conditions: The HT-1080 human fibrosarcoma (ATCC #CCL 121) and the BALB/c 3T3-A31 murine fibroblast (ATCC #CCL 163) cells were purchased from the American Type Culture Collection (Rockville, MD); the B16-F1 murine melanoma cell line was obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing either 10% heat-inactivated fetal bovine serum (FBS) (HT-1080 and B16-F1 cells) or 10% bovine calf serum (BCS) (A31 cells) (Gibco), supplemented with essential and non-essential amino acids, vitamins, L-glutamine, penicillin and streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 93% air and 7% CO_2. To ensure maximum reproducibility, cultures were grown for no more than 6 passages after recovery from frozen stocks and cell viability was monitored by trypan blue dye exclusion.

AMF Purification: AMF was purified as described previously (17,18); briefly, semiconfluent 3-day monolayer cultures of HT-1080 fibrosarcoma or B16-F1 melanoma cells were washed with PBS (2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8mM Na_2HPO_4, pH 7.2) and cultured in protein-free medium. After 24 h the medium was collected and replaced with fresh serum-free medium which was also collected after 24 h. The conditioned medium was clarified by centrifugation (10,000 x *g* for 10 min at 4°C) and dialyzed extensively against water to remove salt and small molecular weight macromolecules. The medium was then concentrated by lyophilization, resuspended in PBS and dialyzed against PBS. Proteins were

separated by molecular sieve chromatography on a Sephacryl S-200 gel filtration column (Pharmacia LKB Biotechnology, Inc.) using PBS as buffer. Fractions were assayed for migratory activity and the single peak of activity was [^{125}I]-labeled and analyzed by one and two dimensional PAGE as described previously (17,18) to verify purity of the isolated AMF.

Phagokinetic Track Motility Assay: Uniform carpets of gold particles were prepared on bovine serum albumin (BSA)-coated glass coverslips according to Albrecht-Buehler as described (27). Colloidal gold-coated coverslips were placed in 35-mm tissue culture dishes and 5,000 cells in suspension culture were added to each plate. After allowing the cells to adhere for 2.5 h, fresh DMEM + BCS was replenished and the cultures were incubated for 1h with or without 200 ng/ml pertussis toxin. Cells were then stimulated and after 24 hours the phagokinetic tracks were visualized using dark-field illumination in a Nikon diaphot inverted microscope at a magnification of 200X. The area cleared by at least 50 cells was measured using JAVA (Jandel Video Analysis, Jandel Corp.) quantitation software and the standard error reflecting a 95% confidence interval was calculated.

Cell Proliferation Assays: *a) Analysis of DNA Synthesis.* A31 cells were plated at equal numbers in 24-well plates, grown for three days and serum starved into quiescence with DMEM + 0.5% BCS. Fresh media with either 0.5% (absence of serum) or 10% (presence of serum) heat-inactivated BCS was replenished and the cells were stimulated with serial dilutions of AMF for 20 hours in the presence or absence of 200 ng/ml PT (Sigma). Cultures were then labeled for 4 hours with [*methyl*-^{3}H]thymidine (1μCi/ml, ICN) and large molecular weight macromolecules were precipitated with a final concentration of 10% trichloroacetic acid. DNA was extracted from the acid-insoluble fraction by heating in 3% perchloric acid at 95°C for 15 min followed by cooling for 15 min at 4°C. Radioactive incorporation was measured by liquid scintillation spectrometry in Ecolume counting cocktail (ICN) using an LKB Rackbeta Model 1209 scintillation counter. Analysis of HT-1080 growth was as above except that FBS was substituted for BCS and serum-starvation was 24-48 hours.

b) Analysis of Cell Number. Cell proliferation was assayed according to time- and dose-dependence essentially as described (28,29). Briefly, 1.5 x 10^4 A31 cells were plated in 0.5 ml 10% BCS media per well of a 24-well plate and after 24 hours the cells were washed three times with PBS and fresh DMEM containing 0.5% BCS and supplements was added. The cells were starved for 24 hours to synchronize the cultures and then stimulated with 0.1 ng/ml AMF for varying times or with AMF at varying concentrations for eight days. Fresh factor was added every two days as described (28) and the cells were quantitated after trypsinization using a particle counter Model Z$_{H}$ (Coulter Electronics, Hialeah, FL). Each condition was assayed in quadruplicate and the value of each well represents the mean of 5 quantitations.

Immunological Detection of gp78: *a) Indirect Immunofluorescence.* The localization of gp78 on the cell surface was performed as described previously (30). Briefly, cells were plated on glass coverslips, grown for three days, washed with PBS containing 4mM calcium, and fixed for 10 minutes with 3.3% paraformaldehyde. After extensive washing, the cells were stained for 30 minutes at room temperature with the anti-gp78 mAb (described in 31). Cover slips were washed further and incubated with FITC-conjugated rabbit anti-rat secondary antibody (Zymed, So. San Francisco, CA) for 30 minutes at room temperature. Controls received either secondary antibody alone or a non-specific rat immunoglobulin. After additional washing, cover slips were mounted in gelvatol (15% polyvinyl alcohol 2000, 30% glycerol in PBS pH 7.2) and visualized using a Nikon optiphot fluorescence microscope.

b) Protein Electrophoresis and Immunoblotting. Analysis of total cellular gp78 was performed essentially as described (30). Briefly, cells were harvested with 0.25% trypsin, washed twice with PBS and equal numbers of cells were suspended in Laemmli sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels (32). Gels were electroblotted to Immobilon-P nylon membranes (Millipore) and the membranes were quenched with 5% non-fat dry milk to block remaining free protein-binding sites. Blots

were incubated with anti-gp78 mAb for 1 hour, washed, and incubated with [^{125}I]sheep anti-rat secondary antibody (ICN). After further washing the blots were processed for autoradiography.

***In Vitro* Monolayer Wound Assay:** The experimental wounding assay was performed according to Folkman and Moscona (33). A31 fibroblasts were grown to confluent monolayers, wounded with a sterile scraper, and washed extensively with sterile PBS containing 4 mM calcium to remove cellular debris. The cultures were then replenished with fresh DMEM containing 10% BCS with or without 50 pg/ml AMF and/or 200 ng/ml PT. Cultures were subsequently fixed with methanol and giemsa-stained at zero time, 24 hours, and 48 hours.

RESULTS AND DISCUSSION

It has been previously demonstrated that 3T3-fibroblasts do not secrete AMF (16); however, in this report we show that they respond in a paracrine fashion to tumor-derived AMF, exhibiting a two- to three-fold increase in motility upon stimulation with either the human fibrosarcoma or murine melanoma AMF (Table 1). Previous studies have shown that an anti-gp78 mAb mimics the effect of AMF, enhancing cell motility (30). Table 1 shows that A31-fibroblasts responded with increased motility upon stimulation with either AMF or the anti-gp78 mAb in a manner similar to that observed in the AMF-producing cells (17,18). This motility-inducing effect was inhibited by PT, suggesting that motility signal transduction of A31 cells in response to AMF and the anti-gp78 mAb proceeds through a PT-sensitive G-protein following binding to gp78 on the cell surface (see below), as described previously for human (19) and murine (17) melanoma cells.

AMF-treatment of A31-fibroblasts stimulated DNA synthesis in a dose-dependent manner (Fig. 1A). Levels of enhanced [^{3}H]thymidine incorporation were similar to those reported for the mitogenic effects of HGF-SF, EGF, TGF-α and the FGF's on human keratinocytes (8) as well as that reported for oncostatin M on AIDS-Kaposi's sarcoma cells (34,35) and smooth muscle cells (36). Maximal response was observed at AMF concentrations of 0.1 ng/ml, with 2.3 to 2.7 times the level of [^{3}H]thymidine incorporated by treated cells than untreated controls ($p < 0.01$ by Student's T-test). Enhanced incorporation of

Table 1. ***Paracrine motility response of 3T3-A31-fibroblasts to stimulation with exogenous AMF.*** A31-fibroblasts were plated directly on gold-coated glass coverslips in the presence or absence of 15 pg/ml AMF or 25 μg/ml anti-gp78 mAb, for a 24 hour phagokinetic migration assay as described in Materials and Methods. The migration of at least 50 cells in each treatment group was analyzed and expressed as the average clearance per cell $\pm$ standard error reflecting a 95% confidence interval.

Treatment	Motility (μm^2/h)
Control	26.2 $\pm$ 4.9
B16-F1 - AMF	59.6 $\pm$ 6.9
HT-1080 - AMF	58.7 $\pm$ 7.8
HT-1080 - AMF + PT	28.9 $\pm$ 3.2
anti-gp78 mAb	59.1 + 8.6

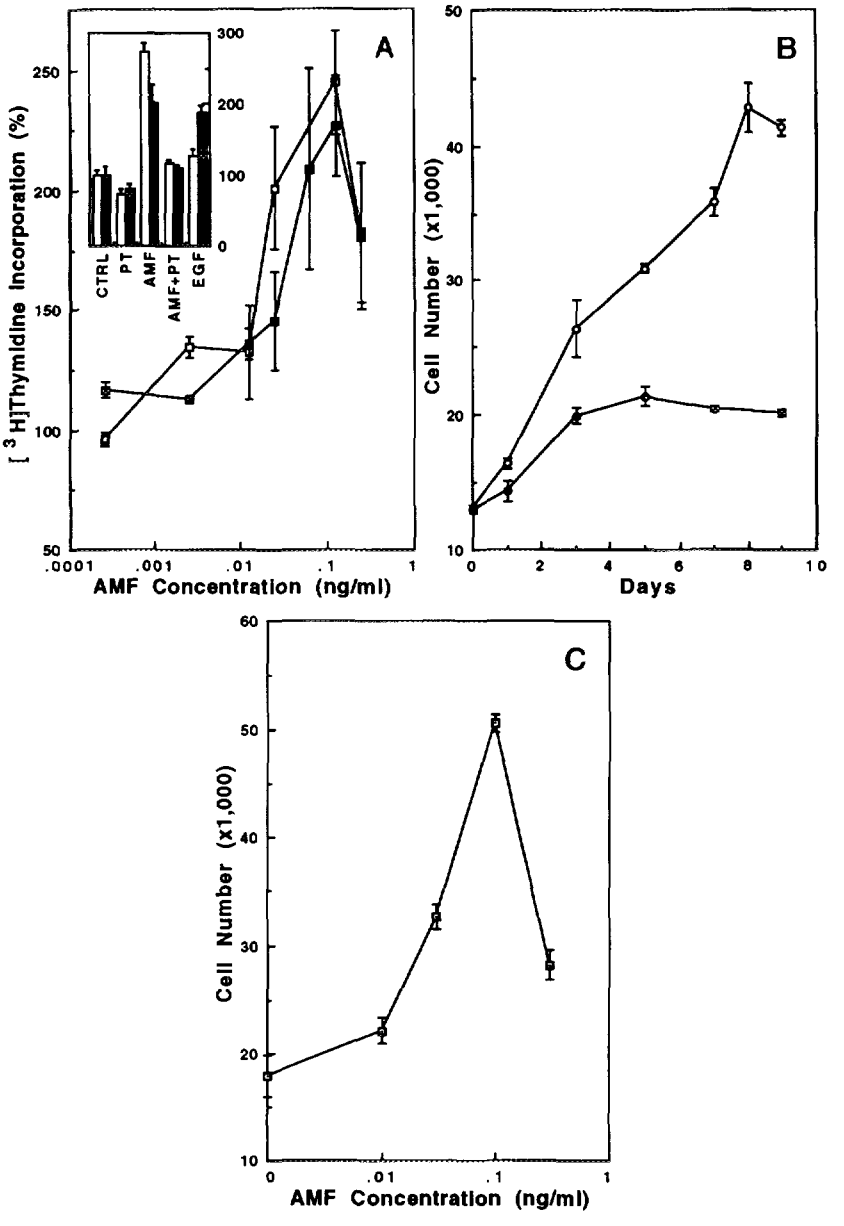

Figure 1. AMF stimulation of 3T3/A31 fibroblast proliferation. (A) Synchronized cells were stimulated with AMF and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Radioactive incorporation is plotted as percent of control $\pm$ SE and represents the mean of triplicate measurements in at least three replicate assays; $p<0.01$ by Student's T-test. (A-*INSET*), pertussis toxin-inhibition of AMF-stimulated DNA synthesis. Cells were prepared as above and stimulated with 0.1 ng/ml AMF in the presence or absence of 200 ng/ml PT; inset axis identical to the main figure; $p<0.01$. EGF, epidermal growth factor control (1 ng/ml). Filled symbols, stimulation in the presence of serum; open symbols, stimulation in the absence of serum. (B) Time course of AMF-stimulated growth. Cells were cultivated with (open circles) or without (filled circles) 0.1 ng/ml AMF for the indicated times and then washed, harvested and counted. (C) Concentration-dependence of AMF-induced A31 proliferation. Cultures were incubated for 8 days with the indicated amounts of AMF and counted as in B. Fresh factor was added every two days and each point represents the mean of quadruplicate experiments.

thymidine into cellular DNA may not be associated with an increase in cell proliferation, therefore we examined the mitogenic effect of AMF using a cell count assay. Cultures treated with 0.1 ng/ml AMF, which evoked maximal [^{3}H]thymidine incorporation, responded with a significant increase in cell number after eight days over unstimulated controls (Fig. 1B). Similarly, endothelial cells stimulated with HGF-SF also show a peak proliferation at 6 to 8 days (28). In addition, the mitogenic effect of AMF on cell proliferation was concentration-dependent in a manner which paralleled the AMF-stimulation of thymidine incorporation (Fig. 1C). AMF's mitogenic effect was similar in the presence or absence of fetal bovine serum (Fig. 1A), implying that at least in this system AMF acts as a competence growth factor. Growth stimulation was blocked by PT (Fig. 1A, inset) at a concentration which inhibits motility induction (17-19), indicating that the mitogenic response to AMF may proceed through a similar signaling pathway as the AMF-induced motility response. Cholera toxin failed to inhibit AMF-stimulated DNA synthesis (data not shown), similar to its lack of effect on AMF-stimulated cell motility (19). The AMF-producing HT-1080 fibrosarcoma cells responded to AMF mitogenic stimulation in a similar manner (data not shown), also exhibiting maximal stimulation of DNA synthesis at 0.1 ng/ml of AMF. Although maximal stimulation was less pronounced in these cells [1.4 to 1.7 fold, ($p<0.01$ from control)], this is presumably due to the inherent difficulty in synchronizing tumor cell cultures for the DNA synthesis assay.

At high doses of AMF a decrease in the mitogenic effect was noted (Fig. 1A and C). This decrease was not due to a toxic effect of AMF on the cells since similar concentrations had no effect on cell viability and continued to stimulate motility (data not shown). This decreased mitogenicity at high concentrations is similar to the effects of EGF and TGF-α on hepatocytes (37), acidic FGF on epithelial bladder carcinoma cells (38), HGF-SF on keratinocytes (8) and endothelial cells (28), as well as EGF and betacellulin, a newly described mitogen from pancreatic β cell tumors, on 3T3 fibroblasts (29), and could be explained by a possible down-regulation of gp78 expression (see below). In addition, the AMF concentration required for maximal mitogenic stimulation is 6 to 10 fold higher than that needed for maximal motility stimulation (Table 1, refs. 17,18), suggesting a concentration-dependent regulation of action similar to that described for acidic FGF on rat bladder carcinoma cells whereby the level of FGF required to increase motility was in excess of the concentration necessary to stimulate DNA synthesis (38). The relatively narrow effective mitogenic concentration range of AMF could represent a concentration-dependent mechanism of target specificity whereby different biological responses might be elicited by different cells under different conditions, allowing a single cytokine to exert more than one function pending the microenvironment.

Morphological changes induced by growth- and motility-regulating factors have been observed in target cells within minutes and include cellular elongation, membrane ruffling and the extension of lamellae and pseudopodia (22). AMF-stimulated fibroblasts exhibited an elongated morphology and enhanced ruffling (Fig. 2B) in comparison to untreated cells (Fig. 2A). This effect was exaggerated in the absence of serum (Fig. 2C), although control cells without serum were generally indistinguishable from controls with serum after 24 hours. This effect resembles the morphological changes described previously in AMF-producing cells which were associated with alterations in the cytoskeletal architecture and migration of these

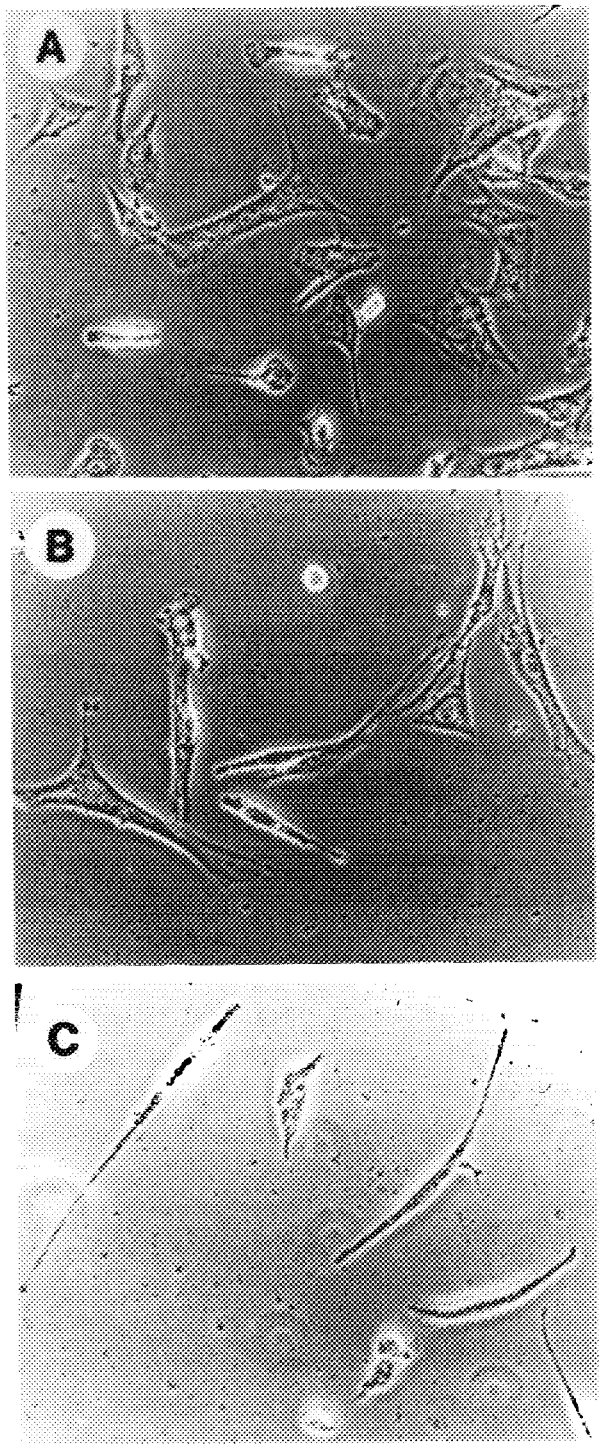

Figure 2. AMF effect on 3T3-A31 morphology. Cells were grown for three days, serum-starved overnight, stimulated with 0.1 ng/ml AMF for 24 hours and fixed with paraformaldehyde. (A) Unstimulated cells in the presence of serum; (B) AMF-stimulated cells in the presence of serum; (C) AMF-stimulated cells in the absence of serum. Representative phase-contrast photomicrographs x320 magnification.

AMF-secreting tumor cells (39), demonstrating that the paracrine effect of AMF on fibroblastic cells is similar to its autocrine effect on tumor cells which produce the factor. Similar morphological changes were observed previously in epithelial cells exposed to HGF-SF (9-11) and in AIDS-Kaposi's sarcoma cells grown in the presence of oncostatin M, recently shown to be a growth-stimulating factor for these cells (34). It is therefore possible that the morphological changes in response to AMF are associated with either enhanced motile activity, DNA synthesis, or both.

The role of cell shape in the control of growth and proliferation of normal A31 fibroblasts has been examined in detail by Folkman and Moscona (33) who demonstrated *in vitro* that increased proliferation of cells in a wounded contact-inhibited monolayer might be regulated by cell shape alterations (cell flattening) along the wound edge. The observation of a higher mitotic labeling index in the flattened cells at the wound edge versus the interior rows supports this hypothesis (33,40). Based on this and the observation that the inhibition of tumor cell growth by differentiating agents is associated with downregulation of gp78 expression and reduced cell motility (41), we questioned whether contact-inhibition of normal A31 fibroblasts is associated at least in part with a loss of motile activity and whether mitogenesis and motility of wound-associated cells in situ might be related to gp78 expression. Localization and quantitation of gp78 in A31-fibroblasts was achieved by detecting anti-gp78 mAb bound to the surface of sparse cultures (Figs. 3a and a') and confluent monolayers (Figs. 3b and b') using indirect immunofluorescence. As observed previously in other cells (30), gp78 is localized to characteristic discrete regions of the A31 cell surface in sparse cultures (Fig. 3a'). Considerably fewer mAbs were bound to the surface of confluent A31 fibroblasts (Fig. 3b'). This down-regulation of surface expression was reflected in a decrease in total cellular gp78 when we analyzed protein extracts of sparse and confluent cultures. Fig. 3c shows that A31-fibroblasts express gp78 protein and that contact-inhibition leads to a greater than 6-fold decrease in the level of gp78 expression, as determined by densitometric scanning. Density-regulated receptor expression is not unique to the A31-gp78 system, as the receptor for acidic FGF is down-regulated in NBT-II epithelial cells cultured at high density (37) and PDGF receptor expression is reduced in contact-inhibited 3T3 fibroblasts as well (42).

Tumor associated mast cells have been implicated in angiogenesis as well as tumor growth and dissemination, and have been shown to respond to tumor-secreted cytokines (43-46). Moreover, mast cells secrete an AMF-like molecule and respond to B16-F1-AMF with increased motility (47). Since mast cells are also involved in wound healing *in situ* (48), we questioned the possible function of AMF in the normal physiological processes of tissue regeneration and repair. To demonstrate the potential for AMF in such a role, we experimentally wounded confluent monolayers of A31-fibroblasts and treated with AMF in the absence or presence of PT (Fig. 4). A single treatment with AMF increased the rate of wound closure by approximately 2-fold over the unstimulated control cultures at 24 hours and 48 hours. Both control and AMF-stimulated wound healing were inhibited by PT. These results support the original finding of Folkman and Moscona (33) who showed that wounding of cell monolayers stimulates cell proliferation which leads to repair; however, the effect of AMF on wound healing adds an additional dimension to this process. As can be seen in Figs. 4B and

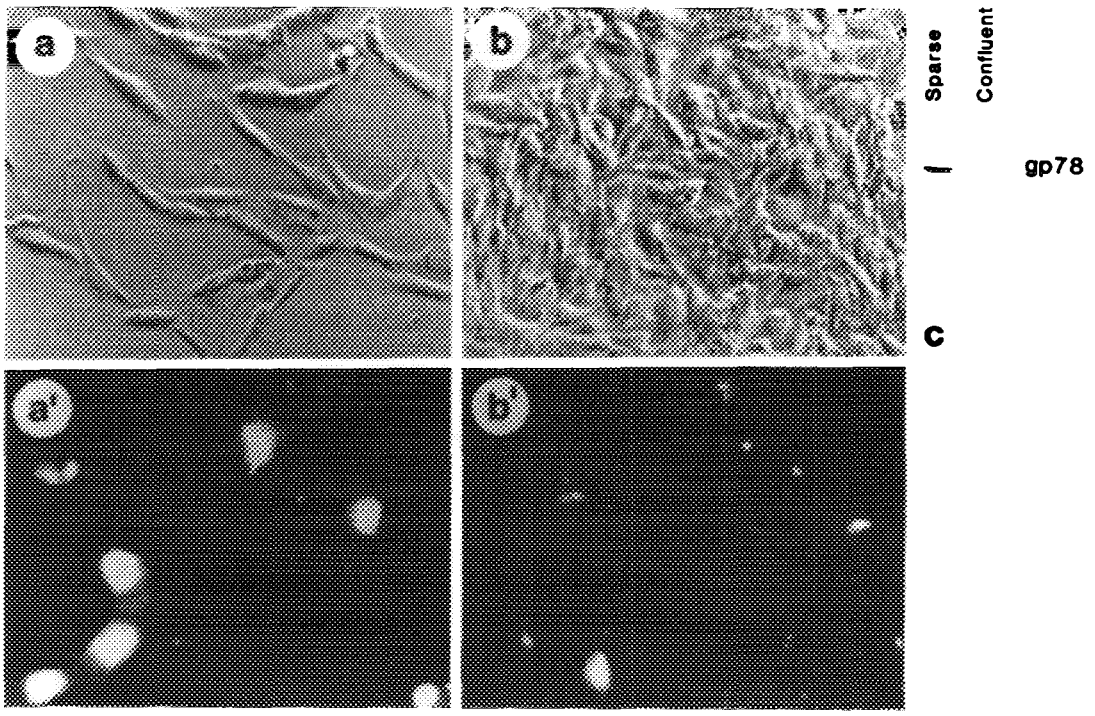

Figure 3. Contact inhibition of gp78 expression. A31 fibroblasts were grown to 25% (a,a') or 100% confluence (b,b') and photographed before (a,b) or after (a',b') surface staining by indirect immunofluorescence with the anti-gp78 mAb as described in Materials and Methods. Representative phase contrast photomicrographs x240 magnification, immunofluorescent photomicrographs x360 magnification. (c) Immunoblot detection of gp78 in sparse or confluent A31 cell cultures, 25% and 100% confluence, respectively.

C, cells in the wound clearing are not confined to the wound edge but may detach from the neighboring contact-inhibited rows and migrate into the cell-free wounded space. Thus, both cell migration and proliferation contribute to the wound-healing process. We assume that the accelerated wound-repair of the AMF-treated cultures may result from AMF's effect on cell motility (Table 1), cell growth (Fig. 1), or both, most likely resulting from an up-regulation of gp78 in the non-density-arrested cells entering the wound (Figs. 3 and 4).

Regulation of proliferation and motility by growth factors and cytokines is critical for proper repair functions in the intact organism. Migration and proliferation of wound-associated fibroblasts, keratinocytes and immune cells have been shown to depend in large part on the actions of growth factors such as PDGF, TGF-α and TGF-β, often involving cross-regulation of one cytokine by another (49-51). In this report we show that AMF is a mitogen which may play a role in wound-healing *in situ*. The source for such AMF activity may be the mast cell, a normal constituent cell of inflammatory and healing responses which is capable of responding to tumor-derived AMF and secretes AMF-like molecules (47). When mast cells aggregate at sites of damage they may release AMF, recruiting fibroblasts into the wound area. The higher concentrations of AMF in the wound proximity may then cause a rapid proliferation of fibroblasts. These cells would subsequently produce matrix components and

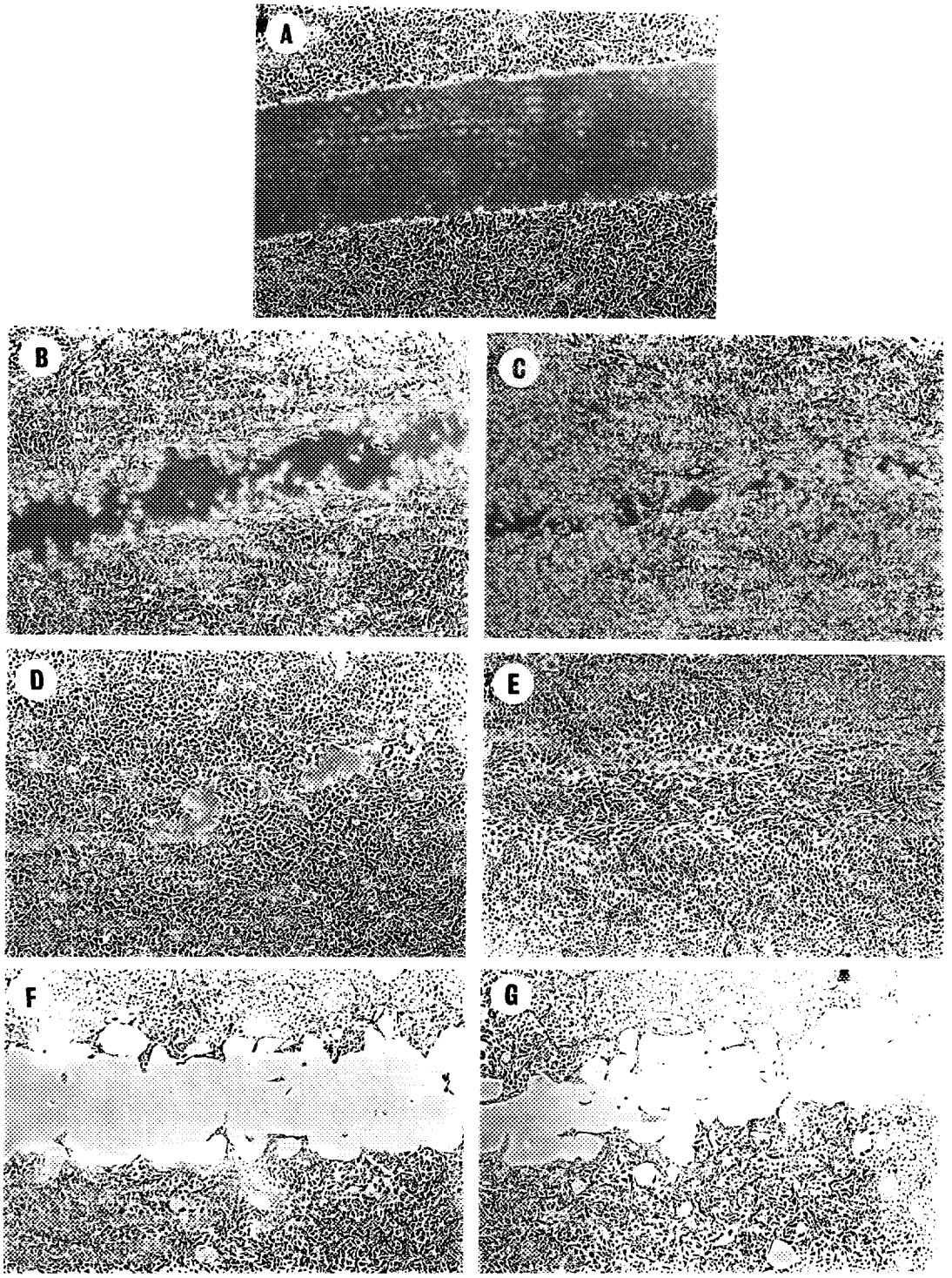

Figure 4. Experimental *in vitro* wounding of confluent 3T3-A31 fibroblast monolayers. Cultures were fixed and stained at zero time (A), 24 hours (B,C) and 48 hours (D,E,F,G) after treatment with 50 pg/ml AMF (C,E,G) or control (B,D,F) in the presence (F,G) or absence (A,B,C,D,E) of 200 ng/ml PT. Representative phase-contrast photomicrographs x35 magnification.

other materials necessary to repair the wound, analogous to the model suggested previously for PDGF (25). Similarly, it has been suggested that mast cells are responsible for the generation of chemotactic leukotrienes that mobilize neutrophils during inflammatory responses (52). In this context and in accordance with the proposal that cancers are wounds which do not heal (53), AMF may represent yet another system whose improper expression or aberrant regulation contributes to the neoplastic or invasive phenotype. These results indicate that future investigations may yield insight into the involvement of AMF as a modulator of cell growth and motility in tissue regeneration or invasion and metastasis. In addition, since AMF acts both as mitogen and motogen, we propose to add the term motility derived growth factor (MDGF) to the definition of AMF.

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