

**HEPARANASE** activity is correlated with the metastatic potential of several cancer cells and is a key enzyme in the breakdown of tissue barriers. It is also involved in the regulation of growth factor and cytokine activity. However, little is known about the factors that induce heparanase in cancer cells. We investigated the effect of three growth factors, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF), on heparanase mRNA induction in lung cancer cells *in vitro*. In addition, we examined the effect of erythromycin (EM) and clarithromycin (CAM), which are 14-membered ring macrolide antibiotics that act as biological response modifiers, on the expression of heparanase mRNA induced by growth factors.

PDGF, HGF and bFGF stimulated cell migration activity and enhanced the expression of heparanase mRNA in the human lung adenocarcinoma cell line A549. Via different mechanisms, EM and CAM modulate the induction by these factors of heparanase mRNA expression on A549 cells. EM also significantly suppressed A549 cell migration induced by PDGF and HGF, and CAM significantly suppressed A549 cell migration induced by bFGF.

The results suggest that the growth factors PDGF, HGF and bFGF are important inducers of heparanase in potentially invasive and metastatic cancer cells. The suppressive effect of heparanase mRNA expression by EM and CAM may have interesting therapeutic applications in the prevention of metastasis.

**Key words:** Heparanase, Lung cancer, Growth factor, Erythromycin, Clarithromycin

## Erythromycin and clarithromycin modulation of growth factor-induced expression of heparanase mRNA on human lung cancer cells *in vitro*

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## Introduction

Cancer cell migration through the basement membranes and degradation of extracellular matrix proteins play an essential role in the various biological and pathological processes in tumor invasion and metastasis.

Histological specimens from lung cancers have documented that central fibrosis and active fibroblast proliferation are among the most important prognostic factors for small bronchioalveolar carcinoma.<sup>1,2</sup> These same studies emphasized the importance of cancer cell-fibroblast interactions in the process of metastasis. These interactions regulate growth factor and matrix metalloproteinase (MMP) production in cancer cells, which leads to the continuation of cell migration.<sup>3–5</sup>

Heparan sulfate proteoglycans (HSPGs) are primarily the chief component of extracellular matrix protein and are also important structures in basement membranes. HSPGs are thought to be involved in cell adhesion, migration, proliferation and angio-

genesis.<sup>6,7</sup> Various classes of enzymes have been reported to play a role in the metastatic process including serine and cysteine protease, MMP and heparanase.<sup>8–11</sup>

Heparanase is an endo- $\beta$ -D-glucuronidase capable of heparan sulfate cleavage, and is reportedly found mainly in platelets, placental trophoblasts, leukocytes, melanomas and hepatomas.<sup>12–15</sup> Recently, overexpression of heparanase in tumor cells was shown to confer a high metastatic potential in experimental mice.<sup>16,17</sup> These reports suggested that heparanase may be the key enzyme in the breakdown of tissue barriers and may be involved in the regulation of growth factors and cytokine activity. However, less is known about the factors that induce heparanase mRNA expression on cancer cells during invasion and metastasis.

Erythromycin (EM) and clarithromycin (CAM) are 14-membered ring macrolide antibiotics that reportedly suppress neutrophil accumulation and effectively treat chronic lower respiratory tract disease.<sup>18</sup> Recent studies have suggested that compounds of this class

limit tumor growth factors.<sup>19</sup> Furthermore, roxithromycin, another 14-membered ring macrolide antibiotic, has been shown to have an inhibitory effect on tumor angiogenesis.<sup>19</sup> These findings suggest that EM and CAM may have anti-metastatic effects on the host-defense system.<sup>20,21</sup>

Consequently, we investigated the effects of growth factors on the induction of heparanase mRNA expression on lung cancer cells *in vitro*. Additionally, we examined the effect of EM and CAM on the expression of heparanase mRNA induced by growth factors *in vitro*.

## Materials and methods

### Materials

Recombinant human platelet-derived growth factor (PDGF)-BB, hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) were purchased from Genzyme (USA). Heparin (porcine), for which activity was  $1 \times 10^3$  U/ml, was purchased from Novo Nordisk (Denmark). EM was provided by Dainavot Pharmaceutical Co., Ltd (Japan), and CAM was provided by Dainihon Pharmaceutical Co., Ltd (Japan). Tissue culture plates were obtained from Sumitomo Bakelite (Japan) and Transwell culture dishes were from Corning Coster (USA). The Matrigel invasion chamber was supplied by Falcon (USA).

### Cell culture

Human lung fibroblasts-1, obtained from Riken Cell Bank (Japan), were cultured in a cell culture flask in modified Ham's F12 (Sigma) supplemented with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO<sub>2</sub> without antibiotics. A549 cells (human lung adenocarcinoma), obtained from Japanese Cancer Research Resources Bank (Japan), were cultured in a cell culture flask in RPMI1640 (Sigma) supplemented with 10% FCS at 37°C in an atmosphere of 5% CO<sub>2</sub>, 95% air without antibiotics. The cells grown to confluence were removed from the culture flask, plated at  $1.0 \times 10^5$  cells in a 35 mm petri dish and incubated with PDGF (2 µg/ml), HGF (4 µg/ml), bFGF (2 µg/ml) and heparin (10 U/ml) without serum and antibiotics for 6 h. Cells were removed from the plates with phosphate-buffered saline and ethylenediamine-tetraacetic acid, replaced and suspended in a micro test tube with 1 ml of F12 or RPMI 1640 media. Cell counts and viability were determined by Trypan blue exclusion (GIBCO) and counted by a hemocytometer. To investigate the effect of EM and CAM, we cultured A549 cells and fibroblasts with EM (10 µg/ml) and CAM (10 µg/ml) for 30 min prior to the addition of the growth factors.

### Cell migration assay

Cell migration induced by PDGF (2 ng/ml), HGF (4 ng/ml), bFGF (2 ng/ml) and heparin (10 U/ml) were measured using the modified Boyden chamber as described previously.<sup>14</sup> The test compounds diluted with Ham's F12 medium were placed in wells in the upper chamber. The wells were covered with Matrigel-coated polycarbonate filters. Human lung fibroblasts ( $5 \times 10^4$  cells) and medium were then added to the wells in the upper chamber. Using the same method as already described, A549 cells ( $5 \times 10^4$  cells) and medium were then added to the wells in the upper chamber. PDGF, HGF and bFGF were added to the wells in the upper chamber for the migration assay. Heparin (10 U/ml) was used as the anti-heparanase compound. After a 6 h incubation at 37°C in 5% CO<sub>2</sub>, the filters were removed and cells were fixed and stained using Diff-Quik stain (International Reagents Co., Japan). Cell migration was determined by counting the number of cells/microscopic field on the lower surface.

### Reverse transcription polymerase chain reaction amplification

Total RNA was extracted from fibroblasts and A549 cells using the acid-guanidinium-phenol method, as per the manufacturer's instructions (AmpliTaq Gold; Roch Molecular System, Inc., USA). First-strand cDNA was prepared from 0.3 µg of total RNA in a 20 µl reaction volume that consisted of 50 mM of Tris (pH 8.3), 75 mM of KCl, 3 mM of MgCl<sub>2</sub>, 10 mM of dithiothreitol, 0.5 mM of dNTPs, 120 U of RNase inhibitor, and 16 U of reverse transcriptase (RT).

Oligonucleotide primers for polymerase chain reaction (PCR) amplification of heparanase were designed according to published DNA sequences for heparanase.<sup>17</sup> The following primers were used:

HPU-380Y, 5'-CGATCCCAAGAAGGAATCAA-3';

HPL-812Y, 5'-TAACTGCGACCCATTGATGA-3'.

Amplification was performed in a final volume of 100 µl with 15 mM of Tris (pH 8.0), 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of dNTPs, 1.0 µmol of each primer and 2.5 U of Taq polymerase. After a "hot-start" (95°C, 10 min), each cycle consisted of 40 sec of denaturation at 94°C, 1 min of annealing at 58°C, and 2 min of elongation at 72°C. Reactions were run for 35 cycles.

### Data analysis

Results are expressed as mean ± the standard error of the mean (SEM). A comparison of the effect of A549 cell migration within experiments was performed by analysis of variance. Statistical significance was established at the  $p < 0.05$  level.

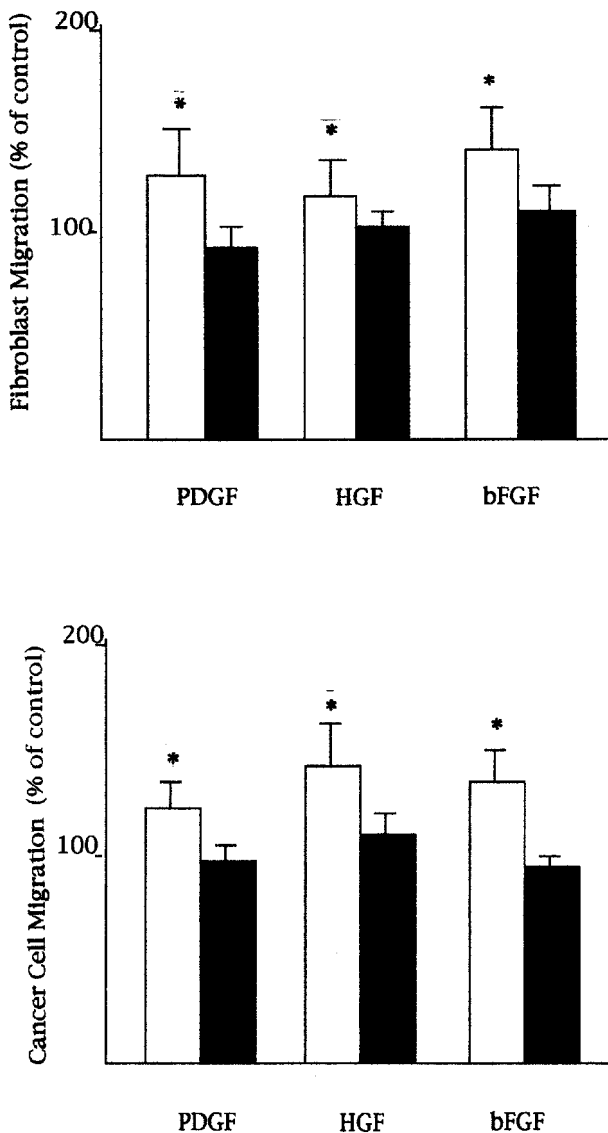


FIG. 1. Cell migration assay in response to PDGF, HGF and bFGF. Bar graph representing the effect of stimulus alone (□) and stimulus in the presence of heparin (■). PDGF, HGF and bFGF induced significantly increased migration in both fibroblasts and lung cancer cells (A549). Heparin inhibited both fibroblast and lung cancer cell migration induced by PDGF, HGF and bFGF. The vertical line represents the SEM of quadruplicate analyses. \*  $p < 0.05$  when compared with controls (no factors).

## Results

### Cell migration assay

PDGF, HGF and bFGF induced significantly increased migration in human lung fibroblasts and A549 cells, whereas heparin significantly inhibited cell migration in both ( $p < 0.05$ ; Fig. 1). EM and CAM did not suppress fibroblast migration induced by PDGF, HGF and bFGF (data not shown).

However, EM significantly suppressed A549 cell migration induced by PDGF and HGF, and CAM significantly suppressed A549 cell migration induced by bFGF ( $p < 0.05$ ; Fig. 2).

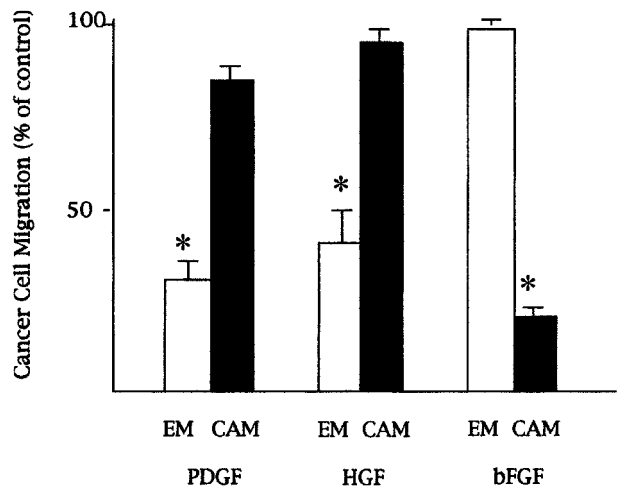


FIG. 2. Bar graph representing the effect of EM and CAM on A549 cell migration induced by PDGF, HGF and bFGF. The vertical line represents the SEM of quadruplicate analyses. \*  $p < 0.05$  when compared with individual growth factor treatment only.

### Effect of growth factors on heparanase mRNA expression in human lung fibroblast

Fibroblasts when cultured with serum-free medium revealed weakly expressed heparanase mRNA, which was not increased by PDGF, HGF or bFGF. Heparin had no inhibitory effects on heparanase mRNA expression (Fig. 3).

### Effect of growth factors on heparanase mRNA expression in A549 cells

A549 cells did not express heparanase mRNA when cultured with serum-free medium. However, growth factors such as PDGF, HGF and bFGF did induce heparanase mRNA expression. Heparin had no inhibitory effects on heparanase mRNA expression (Fig. 3).

### Effect of EM and CAM on heparanase mRNA induced by growth factors in A549 cells

EM suppressed the expression of heparanase mRNA induced by PDGF and HGF, but did not suppress heparanase mRNA expression induced by bFGF (Figs. 4 and 5).

In contrast, CAM suppressed expression of heparanase mRNA induced by bFGF, but not that induced by PDGF or HGF (Fig. 6).

## Discussion

In the present study, we demonstrated that PDGF, HGF and bFGF stimulated cell migration of the human lung cancer cell line. More importantly, these growth factors induced heparanase mRNA expression on the lung cancer cell line *in vitro*.

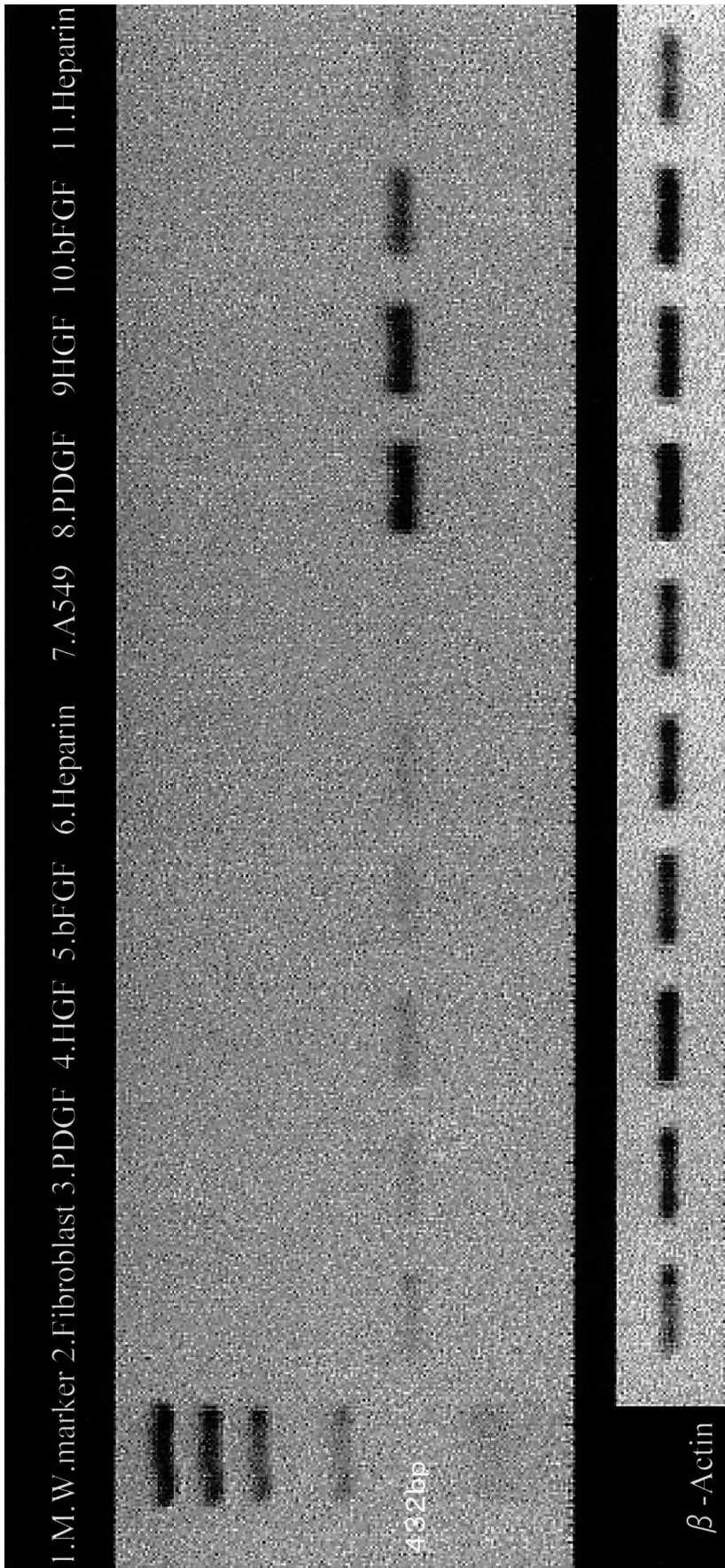


FIG. 3. RT-PCR analysis of heparanase mRNA expression on lung fibroblasts and A549. RT-PCR was performed using oligonucleotide primers specific for human heparanase and the  $\beta$ -actin gene in parallel. Lane 1, Molecular weight marker; lane 2, fibroblast cultured with serum-free medium for control; lane 3, fibroblast stimulated by PDGF; lane 4, fibroblast stimulated by HGF; lane 5, fibroblast stimulated by bFGF; lane 6, fibroblast stimulated by heparin; lane 7, A549 cultured with serum-free medium for control; lane 8, A549 stimulated by PDGF; lane 9, A549 stimulated by HGF; lane 10, A549 stimulated by bFGF; and lane 11, A549 stimulated by heparin.

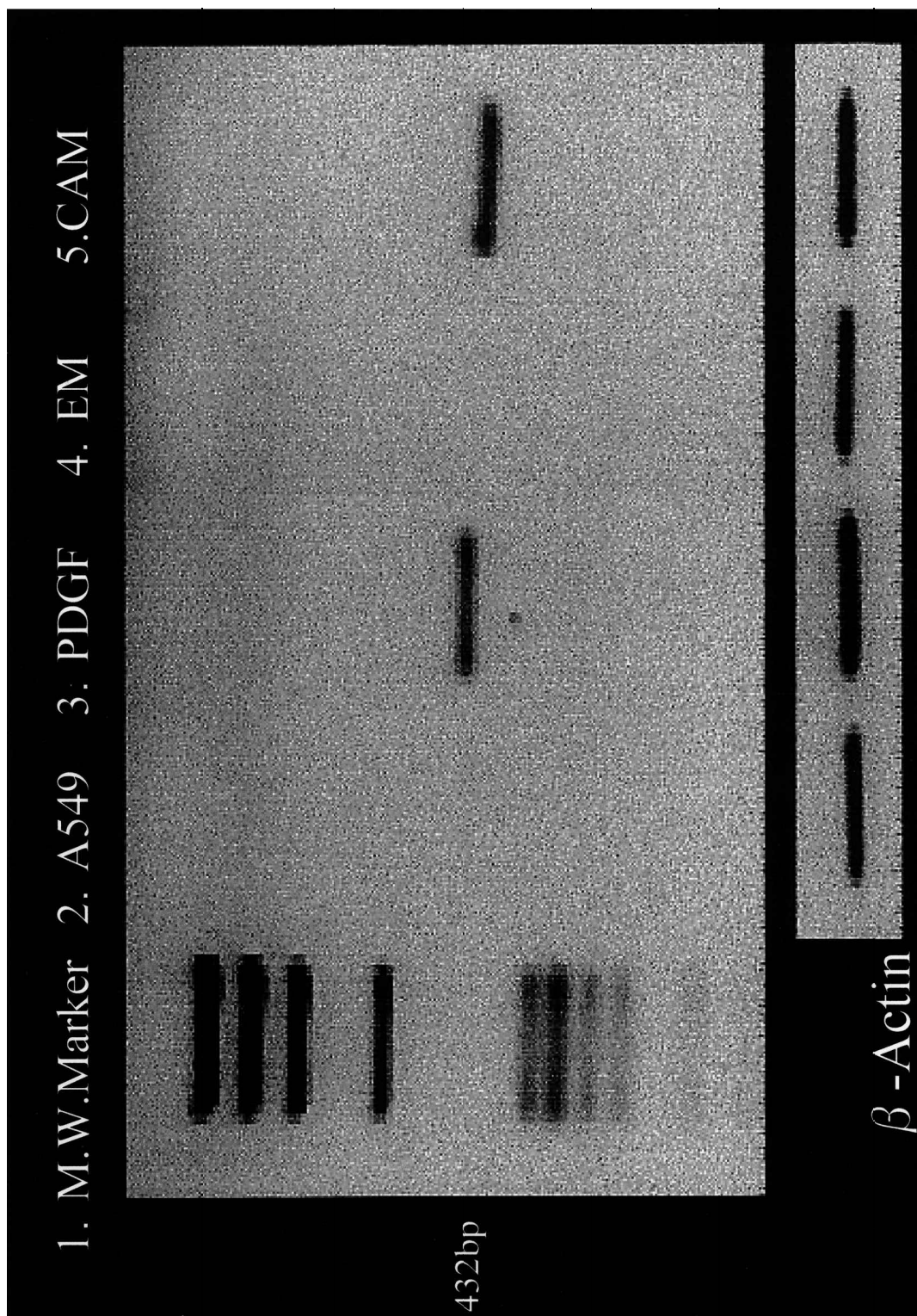


FIG. 4. RT-PCR analysis of heparanase mRNA expression on A549 induced by PDGF. Lane 1, Molecular weight marker; lane 2, negative control; lane 3, positive control; lane 4, effect of EM; and lane 5, effect of CAM.

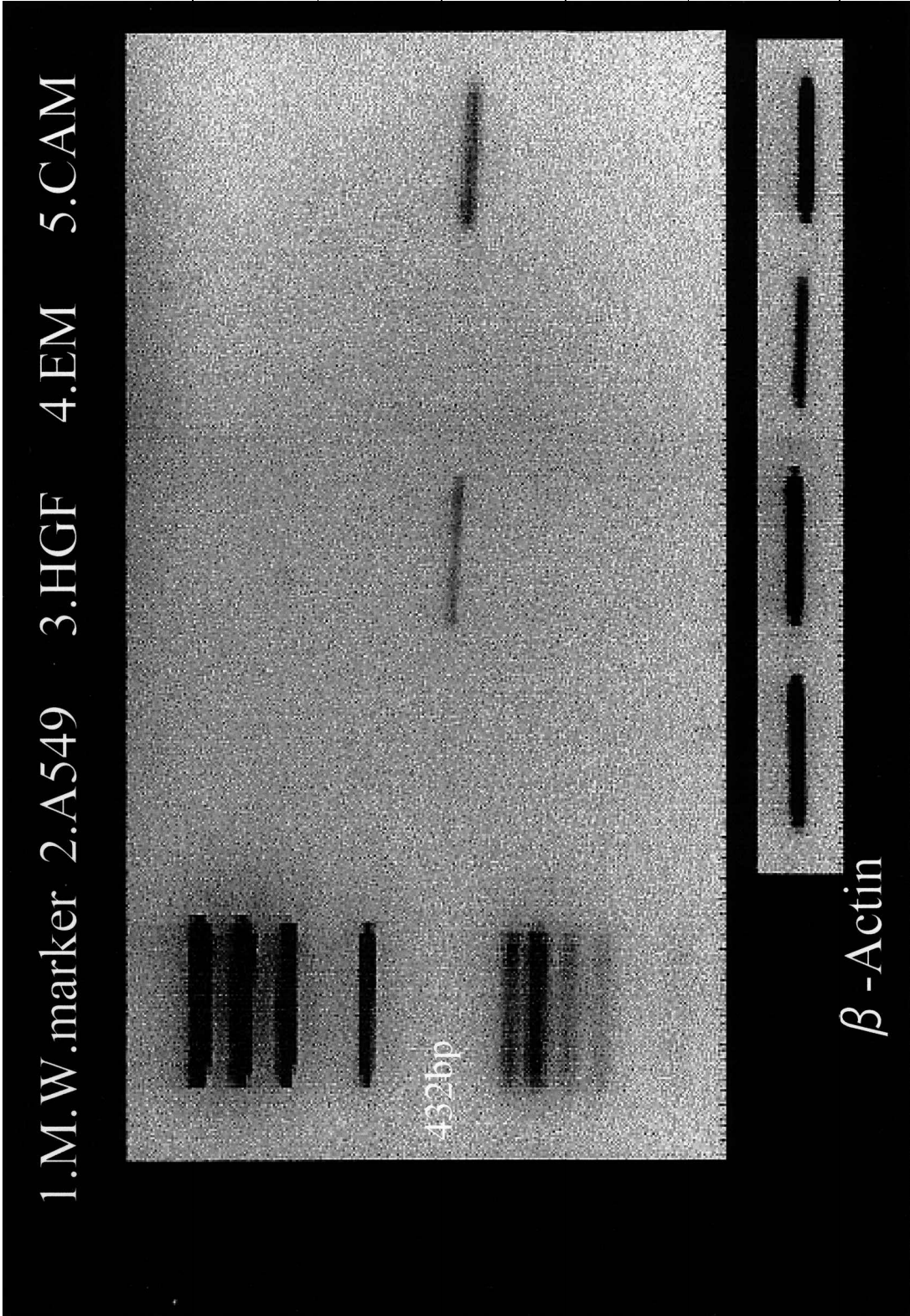


FIG. 5. RT-PCR analysis of heparanase mRNA expression on A549 induced by HGF. Lane 1, Molecular weight marker; lane 2, negative control; lane 3, positive control; lane 4, effect of EM; and lane 5, effect of CAM.

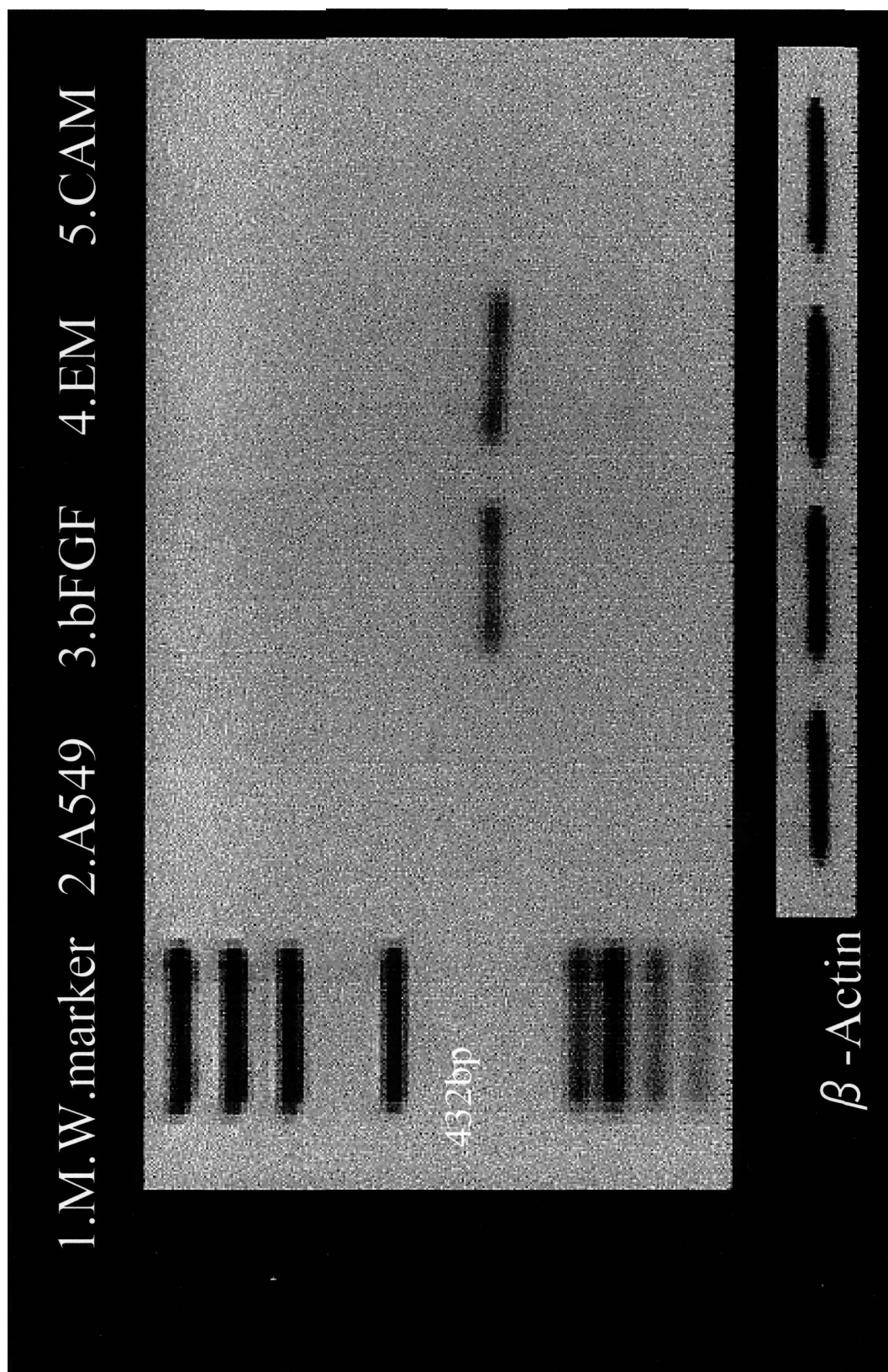


FIG. 6. RT-PCR analysis of heparanase mRNA expression on A549 induced by bFGF. Lane 1, Molecular weight marker; lane 2, negative control; lane 3, positive control; lane 4, effect of EM; and lane 5, effect of CAM.

Tumor cell migration plays an important role in the process of metastasis.<sup>22</sup> Growth factors, including PDGF, HGF and bFGF, and extracellular matrix components are known requirements for cell migration.<sup>23</sup> Furthermore, the existence of PDGF and PDGF receptors is reported to be a prognostic indicator in lung cancers.<sup>24</sup> Produced by several tumor cells, fibroblasts and endothelial cells, bFGF has been implicated in the induction of the angiogenic and stromal responses.<sup>25</sup> Also, it is now recognized that HGF has multiple effects, such as mitogenic, motogenic, and angiogenic effects.<sup>26</sup> These growth factors are produced by tumor and tumor-associated host tissue such as fibroblasts and mesenchymal cells.<sup>27,28</sup> To determine whether growth factors play a biological role in the migration of human lung fibroblasts and cancer cells, we used a cell migration assay employing the modified Boyden chamber method. The cells migrated in a dose-dependent manner (data not shown), suggesting that PDGF, HGF and bFGF influence A549 cells and fibroblast migration. Additionally, PDGF, HGF and bFGF induced heparanase mRNA expression on A549 cells but not on fibroblasts. Our previous study demonstrated that MMP-1 and MMP-9 were strongly positive in the cytoplasm of fibroblasts, and they retained MMP-1 and MMP-2/MMP-9 activity when stimulated by PDGF.<sup>6</sup> More recent studies have demonstrated that heparin and MMP-2 correlate with the proteolytic cleavage sites of fibronectin and anti-adhesive effects.<sup>29</sup> These results suggest that fibroblasts and tumor cells may employ different mechanisms of cell migration. Our data supports the fact that heparanase is inducible in A549 cells by growth factors that also increase migration of these cells *in vitro*.

Our results provide proof that growth factors do indeed play an important role in the induction of heparanase mRNA expression in potentially invasive and metastatic cancer cells. Inhibition of these growth factors, which usually cause increased expression of heparanase, may have a net effect of inhibiting tumor metastasis.

Our data revealed that heparin inhibited growth factor-induced A549 cell migration, which is supported by the fact that heparin is one of the heparanase-inhibitory molecules.<sup>30-33</sup>

Our cell migration assay demonstrated that EM inhibited A549 cell migration induced by PDGF and HGF, whereas CAM inhibited A549 cell migration induced by bFGF. We also found that EM and CAM modulated heparanase mRNA expression on A549 cells induced by growth factors, but in a differential manner. Recent studies have shown that EM enhances natural killer cell activity and increases the survival times of tumor-bearing mice.<sup>34</sup> It has also been found that CAM increases natural killer cell activity and CD8 T-cell cytotoxicity to reduce tumor growth in the murine lung cancer model.<sup>35</sup> We

report here for the first time that EM and CAM suppress heparanase mRNA expression and thus inhibit tumor cell migration. However, further study is required to understand the different mechanisms that occur for EM suppression of PDGF-induced and HGF-induced heparanase mRNA expression, and for the CAM suppression of bFGF-induced expression of heparanase mRNA.

In conclusion, our results provide clear evidence that PDGF, HGF and bFGF growth factors are important inducers of heparanase in potentially invasive and metastatic cancer cells. EM and CAM produce inhibitory effects on growth factors that induce heparanase mRNA expression and may, therefore, have possible therapeutic applications in the future.

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