Journal of Cellular Biochemistry 83:92–98 (2001)

ERK 1,2 and p38 Pathways are Involved in the Proliferative Stimuli Mediated by Urokinase in Osteoblastic SaOS-2 Cell Line

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Abstract Bone metastases from prostate origin generate an osteoblastic reaction that is expressed in vitro by increased osteoblast proliferation. The urokinase-like plasminogen activator (u-PA) present in the media conditioned by tumoral prostatic cells acting as a ligand of the cellular membrane receptor (u-PAR), has been identified as the specific factor that modulates this proliferative reaction. The present study represents an effort to unravel the intracellular pathway by which u-PA activates osteoblastic proliferation and to evaluate the role of cellular receptor u-PAR in this proliferative phenomenon. Our results show that in vitro u-PA stimulates proliferation of SaOS-2 osteoblastic cells by activating the MAP kinase route of ERK 1 and 2 and the p38 pathway. These results are in accordance with the inhibition of intermediate activation and cell proliferation by PD 098059 and SB 203580, specific inhibitors of MEK and p38, respectively. We also show that SaOS-2 cells increase their proliferative response when cells are plated onto vitronectin, the second natural ligand of u-PAR, and that culturing SaOS-2 cells in the presence of u-PA represents a stimuli for u-PAR expression. On the basis of these results we propose that osteoblastic cells respond to the prostate-derived u-PA stimuli in a very efficient manner that includes the utilization of two different signaling routes and the stimulation of the expression of the u-PA receptor. J. Cell. Biochem. 83: 92–98, 2001. © 2001 Wiley-Liss, Inc.

Key words: urokinase; MAP kinase; osteoblasts; metastases

During the late stages of prostate cancer, growth of prostatic cells into bone has been associated with high mortality and represents a leading cause of cancer-related death among males [Koutsilieris et al., 1987]. In vivo, prostate cells cause a sclerotic reaction in the infiltrated bone and, in vitro, prostate-derived factors induce an increase of the proliferative rate of fetal rat calvaria and osteoblastic cell lines [Perkel et al., 1990]. Previous studies in our laboratory have also demonstrated that media conditioned by the human prostatic cell line PC-3 stimulate cell proliferation and blocks cell differentiation [Martínez et al., 1996], as well as decreases the collagen content [Santibáñez et al., 1996] of fetal rat osteoblasts in culture.

The multidomain serine protease urokinase-type plasminogen activator (u-PA) has been implicated as the main soluble factor present in PC-3 conditioned media responsible for this proliferative response [Rabbani et al., 1990]. u-PA exerts its proliferative actions independently of its proteolytic activity. In fact, the inactivated form of u-PA and the amino-terminal fragment of the u-PA molecule (ATF), devoid in proteolytic activity, are able to elicit the mitogenic response [Rabbani et al., 1992]. To stimulate osteoblast proliferation, u-PA binds (by its ATF) in a saturable high-affinity manner to a specific u-PA cellular receptor (u-PAR), a cysteine-rich, highly glycosylated protein that is linked to the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor [Roldan et al., 1990]. It has been reported that ATF is able to induce early response genes such as *fos*, *jun*, and *myc* in the human osteoblastic SaOS-2 cells, phenomenon that is dependent on tyrosine kinase activity [Rabbani et al., 1997]. These results emphasize u-PAR signaling

Grant sponsor: Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) to J.M.; Grant numbers: 897 0028, ENL2000/07; Grant sponsor: DID, Universidad de Chile to J.F.S.; Grant number: I 003-99/2.

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Received 12 February 2001; Accepted 13 April 2001

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DOI 10.1002/jcb.1211

capacity in spite of lacking the cytoplasmic portion. They also suggest the existence of an adapter molecule that links the cell surface with a tyrosine kinase-dependent signaling pathway.

During recent years an increasing body of information has emerged illustrating the importance of the u-PA-dependent signaling capacity in a broad range of biological processes. In addition to the proliferative reaction in the osteoblastic cells, u-PA stimulates cellular motility in human breast cancer and endothelial cells [Nguyen et al., 1998, Tang et al., 1998], stimulates chemotaxis of monocytes [Gyetko et al., 1994], and regulates the adhesion of myeloid cells [Nusrat and Chapman, 1991]. Studies on the u-PA signal-transduction mechanism have led to the conclusion that in the majority of cases the activation of mitogen-activated protein kinases (MAP-kinases) from the extracellular signal-regulated kinases (ERK) family constitute the main route to transmit the membrane receptor-mediated signal to the nucleus [Nguyen et al., 1998, Tang et al., 1998]. It is interesting to note that in spite of the fact that the activation of ERK1 and ERK2 are mainly related to the mitogenic signal [Pages et al., 1993], other biological processes as dissimilar as those mentioned above, can also use this activating pathway.

Another member of the MAPK family, MAPK p38, has also been shown to be specifically required for proliferation in response to growth factor [Raingeaud et al., 1995] and has been mentioned as a target of u-PA-mediated signal transduction in fibrosarcoma cells [Konakova et al., 1998]. At present, the relative contributions of both members of the MAPK family to intracellular signaling through u-PAR has not been defined and constitutes the purpose of this study. Furthermore, although the association of the cellular activity of u-PA with appearance of its receptor is well known, data showing a possible role of the ligand on the expression of u-PAR itself is scarce.

In the present work we show that SaOS-2 cells, a cell line that shares many features with normal human osteoblasts in culture (the capacity to mineralize in culture among others) [Mc Quillan et al., 1995] utilize the MEK and p-38 pathways to signal the proliferative stimulus of u-PA. We show that u-PA-treated cells express more copies of immunoreactive u-PAR, a phenomenon that probably reinforces the maintenance of the u-PA signal and enhances the proliferative stimulus. We also show that the u-PA proliferative stimulus on SaOS-2 cells is enhanced when the cells are plated on Vitronectin (VN) another ligand for u-PAR.

MATERIAL AND METHODS

Materials

High and low molecular weight urokinase, PD 98059 and SB 203580 were obtained from Calbiochem (La Jolla, CA). D-MEM and fetal calf serum (FCS) were purchased from Life Technologies (Rockville, MD). Monoclonal antibodies anti ERK, phospho ERK, p38 and phospho p38 were obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Monoclonal antibody directed to u-PA receptor (CD-87) were purchased on Pharmingen (San Diego, Ca).

Cells

Osteosarcoma SaOS-2 and prostate carcinoma PC-3 were purchased from the American Type Culture Collection (ATCC, Manasas, VA) and were grown in DMEM plus 10% FCS.

Proliferation Assay

In order to test the effect of u-PA on osteoblasts proliferation, SaOS-2 cells were cultured overnight to a density of 3×10^3 cells on 96-well plates (Nunc, Roskilde, Denmark). After this period, cells were maintained for 48 h in a serum-free medium before adding the growth factors. SaOS-2 cells were cultured 48 h with u-PA. In experiments to test the ability of MAP kinase inhibitors (PD 98059 and SB 203580) to block cellular proliferation, these agents, at indicated concentrations, were added 30 min before the respective growth factors.

We also performed experiments where proliferation was assessed in cells plated on different extracellular matrices. 96-well-culture plates were coated with 10 μg/ml of type I collagen (Sigma Chemical, St Louis, MO); 10 μg/ml of Human Fibronectin (Gibco BRL, Rockville, MD); 1 μg/ml of Human Vitronectin (Gibco BRL, Rockville, MD), and 5 μg/ml of Human Laminin (Gibco BRL, Rockville, MD). After an overnight incubation at 4°C, plates were washed with PBS/0.1% BSA and blocked with 1% BSA. Cells were seeded onto these different extracellular matrices for 48 h and then stimulated with 20 nM u-PA for 24 h. After this period,

in all cases, cellular proliferation was measured by immunodetection of BrdU incorporation during DNA synthesis using a kit from Roche, Manheim, Germany according to the manufacturer's instructions.

SDS–PAGE and Inmunoblotting

Semi-confluent cultures of SaOS-2 cells plated on 6-well culture plates (NUNC, Roskilde, Denmark) were treated at different periods with 20 nM u-PA and lysed with a PBS buffer containing 2 mM EDTA, 1% Nonidet, 1mM PMSF, 1 mM NEM, 10 μg/ml Pepstatin, 0.02% Aprotinin, 1 mM Vanadate, and 2 mM β-Glycerol phosphate. Once lysed, proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels and transferred to PVDF membranes. Transfers were blocked for 2 h at room temperature with 3% BSA in TBS 0.5% Tween 20, and then incubated with primary antibodies diluted in the blocking solution. Anti ERK, phospho-ERK, anti p38, and phospho-p38 were diluted 1:1500. After incubation with primary antibody, membranes were washed with TBS 0.3% BSA in 0.05% Tween 20 and afterwards developed using the ECL kit for Western blotting (Amersham, Life Science) according to the manufacturer's instructions.

To detect changes in u-PA receptor molecule after u-PA treatment, cells were incubated 24 h with 20 nM u-PA in serum-free culture medium. After this, cells were detached with EDTA, resuspended in a solution of 0.25 M sucrose in PBS that also contain 1 mM PMSF, 1 mM NEM, 10 μg/ml Pepstatin, and 0.02% Aprotinin, and disrupted by sonication. Large fragments and nuclei were removed and washed by centrifugation at 600g for 15 min at 4°C and the remaining subcellular supernatant was spun at 20,000g for 2 h at 4°C. The resulting pellet, after two washes in PBS was resuspended in 0.1% Triton X-100 plus the above mentioned protease inhibitors and were used in Western blot analysis. Proteins was measured using the Bio-Rad detergent compatible protein assay (Hercules, CA).

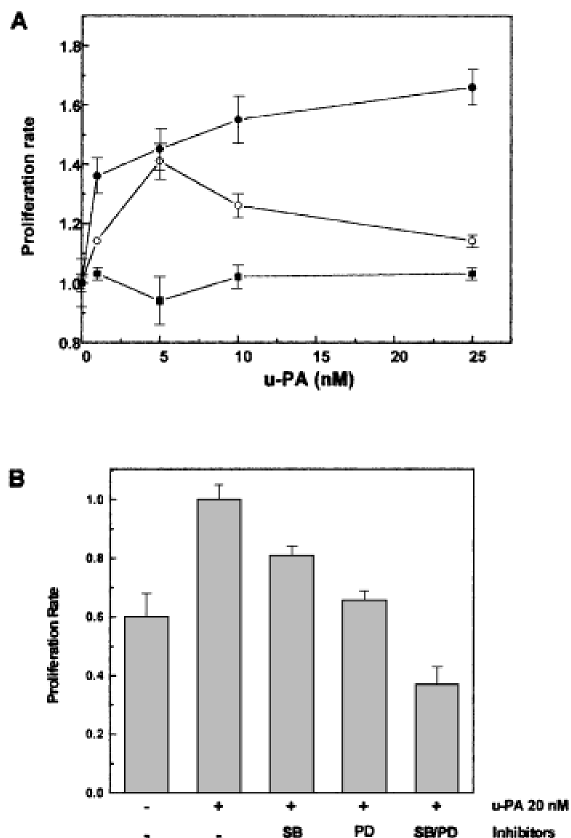

Fig. 1. **A:** Inhibitory effect of PD 098059 and SB 203580 on SaOS-2 cells proliferation stimulated by u-PA SaOS cells (3×10^3) were seeded in 96-well plates and stimulated to growth in the presence of increasing concentrations of u-PA. Proliferation was measured according Materials and Methods. Symbols: Full circles: cells stimulated by u-PA. Empty circles: u-PA plus 10 μM SB 203580. Full squares: u-PA plus 25 μM PD 098059. The results were expressed relative to the proliferative rate of cells cultured in the absence of stimuli and inhibitors and represent the average of three independent experiments with each treatment run in triplicate. The bars indicate standard deviation. **B:** Synergistic effect of PD 098059 and SB 203580 on SaOS-2 cells stimulated to growth with u-PA. SaOS-2 cells (3×10^3) stimulated to growth with 20 nM u-PA were treated with 10 μM SB 203580 (SB) or 25 μM PD 098059 (PD) or the mixture of both agents (SB/PD). Proliferation was measured as described in Materials and Methods and expressed relative to the proliferative rate reached at 20 nM u-PA in the absence of inhibitors. Values represent the median of two different experiments with each treatment run in triplicate ($n = 6$). The bars indicate standard deviation.

RESULTS

Effect of MAP Kinase Inhibitors PD 098059 and SB 203580 in u-PA- Stimulated SaOS-2 Proliferation

u-PA increased in a dose-dependent manner the proliferative rate of serum-free cultured SaOS-2 cells (Fig. 1A). When SaOS-2 cells were stimulated to grow in the presence of PD 098059, a selective inhibitor of MEK, growth inhibition was almost complete over the entire concentration range of the proliferative stimulus. Conversely, using SB 203580, a specific p38 MAP kinase inhibitor, the u-PA-dependent

proliferation was only partially inhibited suggesting that the p38 MAP kinase signaling pathway is indeed involved in the proliferative response. No stimulation was obtained in any experiment when cells were exposed to Low Molecular Weight Urokinase (33 kD), expressing only the proteolytic activity (data not shown).

In order to gain further insight into the functional role of both signal transduction pathways, we stimulated SaOS-2 cells with u-PA at concentrations that induce a significant effect (10 nM) and treated cells with 10 μM SB 203580, 25 μM PD 098059, and the mixture of both inhibitors. As Figure 1B shows, the mixture of both inhibitors displays a synergistic effect on the inhibition of u-PA stimuli. Interestingly, the mixture of both inhibitors reduced the proliferation rate to below control values. These results strongly suggest that the proliferative signaling of u-PA in SaOS-2 cells-include the activation of both p38 and ERKs pathways.

Participation of MAP Kinase Pathways on u-PA Stimulated Proliferation of SaOS-2 Cells

In order to study the participation of MAP kinase pathway on the proliferative stimulation mediated by u-PA, we analyzed the time course of phosphorylation of ERK 1 and 2 and p38 of SaOS-2 cells. For this purpose, we incubated cells with 20 nM u-PA and measured the phosphorylated products over a period of stimulation for up to 60 min. u-PA induces a phosphorylation of p42 and p44 with a maximum increase of 2.5-fold at 15 min (Fig. 2A). Under the same experimental conditions a phosphorylation of p-38 occurs with a maximum of 7.7-fold at 30 min (Fig. 2B). In both cases u-PA induces a transient phosphorylation which is not attributable to changes in the expression of the non-phosphorylated form of ERK or p-38.

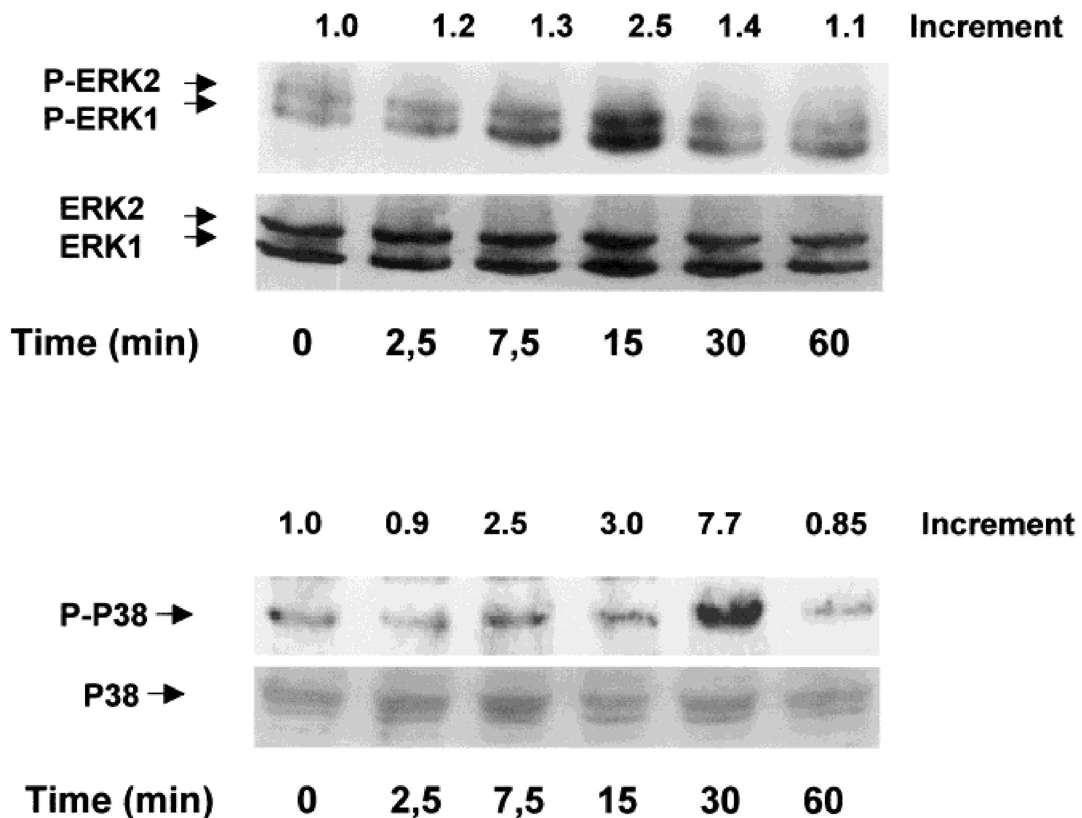

Fig. 2. Time-course of ERK 1,2 and p38 activation in response to u-PA. Semi-confluent cultures of SaOS-2 cells were treated with 20 nM u-PA for 0, 2.5, 7.5, 15, 30, or 60 min. Cell extracts were prepared and an equal amount of proteins were analyzed by SDS–PAGE and immunoblotting with antibodies specific to phosphorylated and total ERKs and p-38 according to Material and Methods. The intensity of individual bands were measured by densitometry and increments corresponds to the rate among the phosphorylated and total immunoreactive protein.

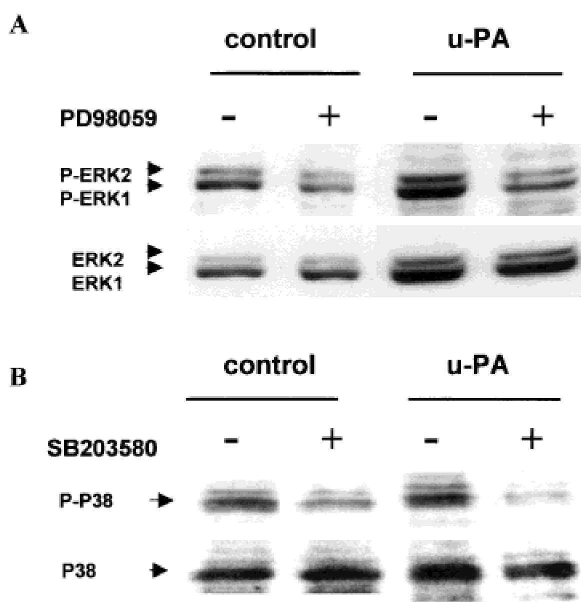

Fig. 3. Effect of PD 098059 and SB 203580 on ERK 1,2 and p38 activation by u-PA. Semi-confluent cultures of SaOS-2 cells were treated for 15 min with 20 nM u-PA with or without PD 098059 (**A**) or treated for 30 min with or without SB 203580 (**B**). Afterwards, cells were lysed and an equal amount of proteins were analyzed by SDS–PAGE and immunoblotting with antibodies specific for activated and total ERKs and p38.

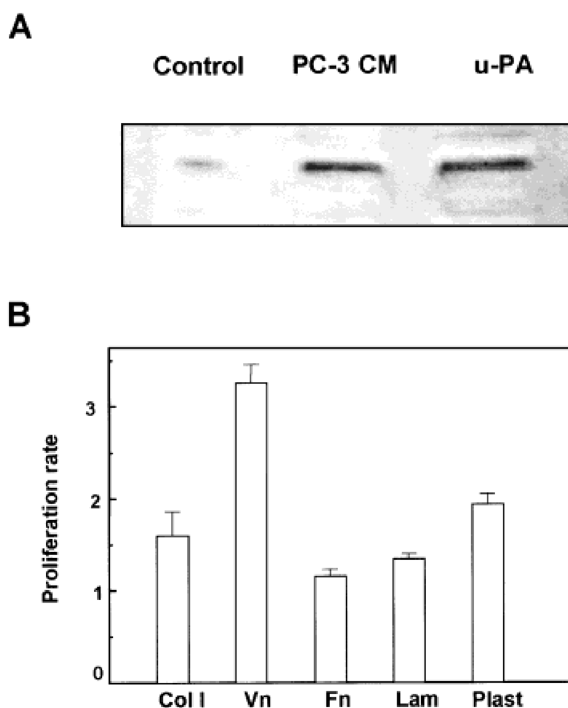

Fig. 4. **A:** Effect of u-PA on the expression of u-PAR. Semi-confluent cultures of SaOS-2 cells were treated for 24 h with media conditioned by PC-3 cells or with 20 nM u-PA in serum-free culture medium as indicated above. After this, cells were detached with EDTA and disrupted by sonication. Membrane fraction was prepared as describing in Materials and Methods and immunoreactive u-PAR was detected by Western blot, as described. An equal amounts of protein (40 μg in 50 μl) was loaded en each lane. **B:** Effect of extracellular matrices on SaOS-2 cell proliferation, SaOS-2 cells (3×10^3) were seeded in 96-well plates previously coated with different matrices: Type I collagen (Col I), Vitronectin (Vn), Fibronectin (Fn), Laminin (Lam), and stimulated to grow in the presence of 20 nM u-PA. Proliferation was measured according to Materials and Methods, and expressed as the proliferation rate of cells stimulated by u-PA plated in each ECM molecule refered to their proliferation rate in the absence of u-PA. Results represent an average of two different experiments with each treatment run in triplicate ($n = 6$). The bars indicate standard deviation.

Effect of MAP Kinase Inhibitors PD 098059 and SB 203580 u-PA-Stimulated ERK and p38 Phosphorylation

To confirm that the u-PA-dependent growth stimulation included the activation of MAP kinase pathway, we measured the inhibition of ERK 1 and 2 and p38 phosphorylation by the specific inhibitors PD 098059 and SB 203580. In these experiments, SaOS-2 cells were induced to grow under the stimuli of u-PA utilizing the incubation time in which the maximum activation was reached from the time-course experiment. Under the stimulus of u-PA ERK and p38 phosphorylation were sensitive to inhibition by PD 098059 and SB 203580 (Fig. 3).

Effect of u-PA in u-PAR Expression and ECM-Associated Proliferative Activity

To analyze whether the contact of u-PA with a u-PA receptor-expressing cell have a functional consequence in the expression status of the receptor, we incubated SaOS-2 cells for 24 h with u-PA or media conditioned by PC-3 cells, a well known source of u-PA from prostatic origin [Martinez et al., 1996]. Previous incubation of SaOS-2 cells with either media conditioned by PC-3 cells or 20 nM u-PA show a significant enhancement of immunoreactivity attributable to a increased expression of the receptor (Fig. 4A).

To assay the functionality of u-PAR in SaOS-2 cells and to test whether the interaction with VN (another ligand of u-PAR) predisposes osteoblastic cells to proliferate under the stimuli of u-PA, we plated SaOS-2 cells onto a variety of extracellular matrix (ECM) molecules and stimulated them with 20 nM u-PA. As Figure 4B shows, cells plated onto VN show a significant and specific increase in proliferation compared with other molecules and plastic

alone. No significant differences in proliferation were observed when cells were plated on different matrices in the absence of u-PA (data not shown). These results suggest the existence of a cooperative phenomena where both ligands of u-PAR (u-PA and Vn) are involved in cell proliferation.

DISCUSSION

In prostate cancer the lodgment of tumoral cells into the bone induces a proliferative response that is not attributable to IGF-I, the physiological effector of osteoblast proliferation [Perkel et al., 1990]. Pioneer studies of Rabbani et al. [1990] established that the High Molecular Weight u-PA molecule present in the media conditioned by the tumoral prostatic PC-3 cells was responsible for this proliferative reaction in osteoblastic cells. Here we study the signal transduction pathways that mediate the proliferative u-PA stimulus, the effect matrix proteins in u-PA-stimulated cellular proliferation and the effect of the ligand on the expression of its own cellular receptor.

Mitogen-activated protein kinases (MAPKs) are part of a group of protein serine/threonine kinases that are involved in cellular signaling [Davis, 1993]. The MAPK family not only includes ERK members, but also JNK and p38 MAP kinases, all of them operating in an integrated network of signal transduction pathways. It has been proposed that external proliferative stimulus signaling is mainly by the ERK pathway [Pages et al., 1993], while the activities of JNK and p38 have been predominantly associated with stress and the action of inflammatory cytokines [Raingeaud et al., 1995]. However, recent reports have demonstrated that p38 displays a broader range of biological activities, including erythroid differentiation [Nagata et al., 1998], cell hypertrophy [Wang et al., 1998], neuronal differentiation [Morooka and Nishida, 1998] and also, cellular proliferation [Maher, 1999]. To date, the association of u-PA signaling with p38 has been scarcely documented in one descriptive study of molecular targets of u-PA-mediated signal transduction [Konakova et al., 1998].

From results presented here, we propose that the human osteoblastic SaOS-2 cells respond to the proliferative stimulus of u-PA by means the activation of ERK and p38 pathways. The participation of these route on the proliferative response of osteoblastic cells was confirmed by measuring the blocking effect of PD 098059, specific inhibitor of MEK, and SB 203580 inhibitor of p38, either in cell proliferation or in ERK and p38 activation, respectively. Data showing that inhibition of proliferation by PD 098059 abolishes almost completely u-PA stimulated proliferation at any concentration of the ligand (Fig. 1A) suggest that the ERK pathway is the main functional route for the u-PA proliferating stimulus. However, from results that show that the mixture of SB 203580 and PD 098059 generate an additive inhibitory response we conclude that both ERK and p38 pathways are operative during u-PA stimulation (Fig. 1B). Moreover, the time course of activation of ERK and p38 by u-PA (Fig. 2) shows that the point of maximum activation with respect to the zero time was earlier for ERK and higher for p38. From the analysis of results in Figure 3 we also conclude that both routes are available even at basal level, because inhibitors affect activation of ERK 1, 2, and p38 in the absence as well as in the presence of external u-PA stimuli. This also was suggested from the results of Figure 1B which show that the mixture of both inhibitors not only abolished the proliferative stimulus of u-PA but the proliferation rate of non-stimulated cells as well. Recent data demonstrating that in tumoral breast cells the expression of both u-PA and u-PAR require the constitutive activity of the p38 pathway provides support to our findings [Huang et al., 2000].

Previous work in SaOS-2 cells have demonstrated that u-PA induces the expression of early response genes such as *c-fos*, *c-jun*, and *c-myc* in experimental conditions similar to those which promote cell proliferation in a phenomenon depending on the binding of u-PA to its receptor [Rabbani et al., 1997]. Our results also show (Fig. 4B) that another natural ligand for u-PAR, Vitronectin, enhances the proliferative response of SaOS-2 cells to u-PA suggesting that u-PAR in contact with Vitronectin may acquire a specific conformation that allows for a more efficient response to the proliferative stimulus of u-PA. Results in Figure 4A also show that a 24 h preincubation of SaOS-2 cells with u-PA induce the expression of more copies of immunoreactive u-PAR on the cell surface, a phenomenon that also could explain the enhancement of the proliferative response mediated by u-PA. It is important to note that the media

conditioned by PC-3 cells, the original source of u-PA that defined its proliferative stimuli on osteoblastic cells, also shows a potent stimulus on the expression of u-PAR. From our results we cannot confirm if the augmented concentration of u-PAR on the cell surface after u-PA treatment is the result of a specific stimulus at transcriptional level or represent, increased stability of the protein molecule.

Taken together our results suggest that u-PA act as a ligand of its cellular receptor, u-PAR, to stimulate cellular proliferation in SaOS-2 cells, depends that u-PA action on the activation of ERK 1, 2, and p38 signaling pathways and that it increases the level of expression of u-PAR. We suggest that the stimulus of dual signaling pathways as well as the amplification of ligand binding sites by u-PA in these cells assures the persistence of the proliferative stimulus.

ACKNOWLEDGMENTS

Authors thank Dr. I. Atwater and C. Rojas for helpful discussions and critical reading of the manuscript.

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