Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 380-386

VASCULOTROPIN/VASCULAR ENDOTHELIAL GROWTH FACTOR INDUCES DIFFERENTIATION IN CULTURED OSTEOBLASTS

Véronique Midy and Jean Plouët@

Laboratoire de Biologie Moléculaire Eucaryote, UPR 9006 CNRS,
118 Route de Narbonne, 31062 Toulouse, France

Received January 13, 1994

SUMMARY : Vasculotropin/vascular endothelial growth factor (VAS/VEGF) is an angiogenic growth factor whose biological activity seems to be restricted in vitro to vascular endothelial cells. We describe here that fetal bovine osteoblasts (OB) bind VAS/VEGF but do not proliferate upon its addition. However VAS/VEGF induces migration, PTH-dependent cAMP accumulation and alkaline phosphatase increase when added to OB. The maximal effects reach levels comparable to that obtained with bone morphogenetic protein 2 (BMP-2), although the VAS/VEGF concentrations required are at least 100 fold lower. Our results suggest that VAS/VEGF could be an important regulator of osteoblastic differentiation.

Vasculotropin (VAS) is a recently identified growth factor angiogenic in vitro as well as in vivo (1). It is also known as Vascular endothelial growth factor (VEGF, 2) or Vascular Permeability Factor (VPF,3). The human gene coding this angiogenic factor has been cloned and sequenced (4,5). Alternative splicing of the mRNA generates three molecular species corresponding to 121, 165 and 189 aminoacids (6). Its biological activity is so far known as restricted to vascular endothelial cells (1-5) and Interleukin 2 stimulated human lymphocytes (7). However endothelial cells cultured from corneas (8) or monocytes (9) bind, migrate but do not proliferate upon VAS/VEGF addition. We describe here that fetal bovine osteoblasts bind VAS/VEGF on an apparent single site and can migrate or increase their alkaline phosphatase or parathyroid hormone dependent cAMP content when exposed to low doses -in the pM range- of VAS/VEGF.

@ Corresponding author. Fax: 61-33-58-86.

0006-291X/94 $5.00

MATERIALS AND METHODS

Growth factors and cell culture conditions : Human recombinant VAS/VEGF 165 was expressed in a baculovirus expression system as described (10). Human recombinant bFGF and BMP-2 were generous gifts of Dr Prats and Wang. Bovine osteoblasts were isolated from fetal calvaria and cultured as previously described (11) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf Serum (FCS), at 37°C in an humidified atmosphere containing 10% CO2. For all experiments, cells were used at passage numbers between 2 and 5.

Binding assays : VAS/VEGF 165 was iodinated using chloramine-T reagent. Subconfluent cells in 6 multiwell plates were incubated with the desired concentrations of iodinated VAS/VEGF in a final volume of 500 µl of binding buffer pH 7,4 (DMEM containig 20 mM Hepes and 1 mg/ml gelatin) for 3 hours. Non specific binding was obtained in parallel dishes incubated with 500 ng of unlabeled VAS/VEGF. Apparent dissociation constant and number of binding sites per cell were determined by the Scatchard's analysis using the Munson's program, as described (12).

Cell proliferation and migration assays : Subconfluent OB cultured in 24-well cluster plates were growth-arrested by serum removal. 30 hours later increasing concentrations of growth factors were added. 20 hours later 1 µCi of ^{3}H thymidine (specific activity 47 Ci/mmole) was added for 4 hours to the wells and the trichloracetic acid precipitable material counted in a scintillation counter. For migration assays, OB cells were trypsinized and 50,000 cells deposited in the upper tank of a Boyden assay chamber (diameter 3 mm). The lower tanks were replenished by DMEM containing the desired concentrations of growth factors and the chambers placed at 37°C 6 hours. The cells which had migrated in the lower chamber were counted with a cell counter after 6 hours.

Osteoblastic differentiation assays : Confluent OB were additioned every other day with or without the desired concentrations of growth factors for 6 days. Cell monolayers were rinsed and further lysed for alkaline phosphatase determinations using para nitrophenol as substrate (13). Parallel dishes were incubated 20 minutes in serum free DMEM suplemented with 1 mM 3-isobutyl-1-methylxanthine in presence or not of 400 ng/ml of parathyroid hormone (PTH 1-34). The cells were further extracted as described (14). cAMP was measured by a commercially available radioimmunoassay (Amersham). Cells were counted at the end of growth factors exposure.

RESULTS

Binding sites for VAS/VEGF were characterized in experiments studying the concentration dependence of the binding of ^{125}I VAS/VEGF to subconfluent OB. The binding was saturable with a half maximal and maximal binding occuring at 9 and 80 pM respectively. Scatchard's analysis of the data of the isotherms of binding revealed one binding site with an

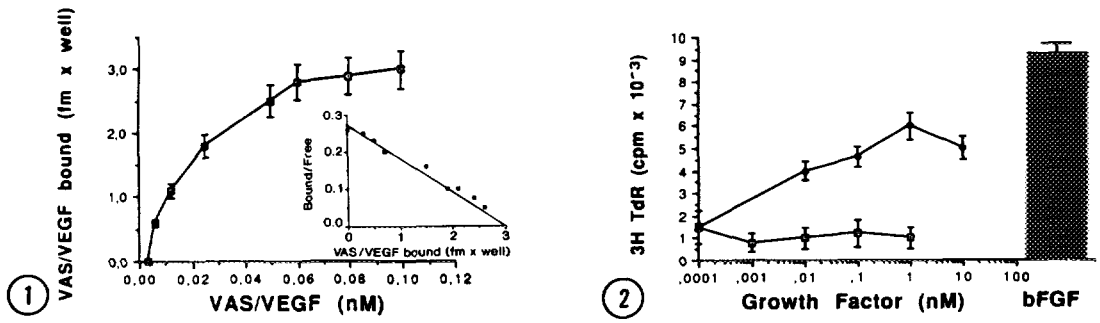


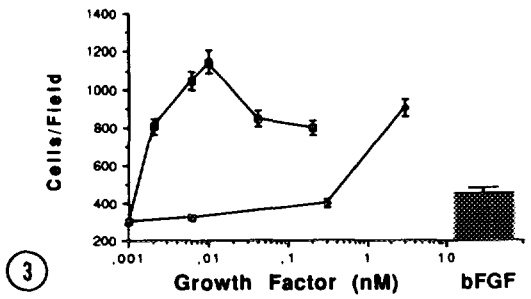
Figure 1. Concentration dependence of iodinated VAS/VEGF binding to OB cells. Apparent dissociation constant and number of binding sites per cell were determined by Scatchard's analysis using the Munson's program (inset). Data are representative of 3 experiments.

Figure 2. Dose response curve of ^{3}H thymidine incorporation into DNA of OB cells between 20 and 24 hours after stimulation with VAS/VEGF (▣), BMP-2 (◆) or bFGF. Data are the means +/- SEM of triplicate dishes and are representative of 6 experiments.

apparent Kd of 12 pM assuming an apparent molecular mass of 45,000 Da for VAS/VEGF. The abscissa intercepts in this representation indicated the presence of 1200 sites per cell (Figure 1)

OB cells incubated with various concentrations of VAS/VEGF did not show any significant change in their DNA synthesis whereas bFGF and BMP-2 acted as potent mitogens as expected (Figure 2). In another set of experiments OB migration was stimulated by VAS/VEGF 8 fold upon untreated cultures in a dose dependent manner. The optimal effect was achieved with a concentration of 1 pM of VAS/VEGF or 120 pM of BMP-2 (Figure 3). bFGF had no action.

Alkaline phosphatase and PTH dependent cAMP production were assayed as markers of osteoblastic differentiation on confluent OB. As shown on Figure 4 VAS/VEGF and BMP-2 increased 6-8 fold the alkaline phosphatase activity. However half-stimulation was achieved for 0.7 and 400 pM respectively. Neither bFGF nor BMP-2 affected cAMP production in absence of PTH 1-34 whereas VAS/VEGF alone stimulated cAMP. The ratio of cAMP production in untreated as compared to PTH 1-34 treated OB increased by 1.9 (n=5) indicating that OB retained in culture an osteoblastic phenotype. VAS/VEGF and BMP-2 increased the cAMP production in response to PTH 1-34 by 2.3 and 2.9 fold respectively (Figure 5) whereas bFGF



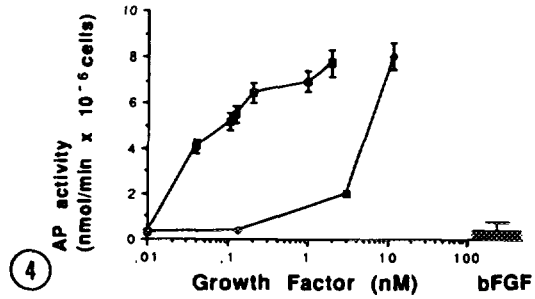


Figure 3. Dose response curve of OB cells migration in a Boyden's chamber assay exposed 6 hours to the presence of VAS/VEGF (□), BMP-2 (◆) or 1 nM of bFGF. Data are the means +/- SEM of quadriplicate dishes and are representative of 3 experiments.

Figure 4. Dose response curve of alkaline phosphatase activity of OB cells exposed 6 days to VAS/VEGF (□), BMP-2 (◆) or 1 nM of bFGF. Data are the means +/- SEM of triplicate dishes and are representative of 6 experiments.

had no effect. None of the assays provided evidence that BMP-2 and VAS/VEGF could act synergistically (not shown).

DISCUSSION

VAS/VEGF is an angiogenic growth factor which binds to specific cell surface receptors and thereby induces proliferation and migration of vascular endothelial cells. Our findings indicate for the first time that this growth factor also triggers the differentiation of cultured osteoblasts.

Binding studies have demonstrated that VAS/VEGF binds to OB cells in a saturable and specific manner. Analysis of the data by the Scatchard's procedure favoured the existence of a

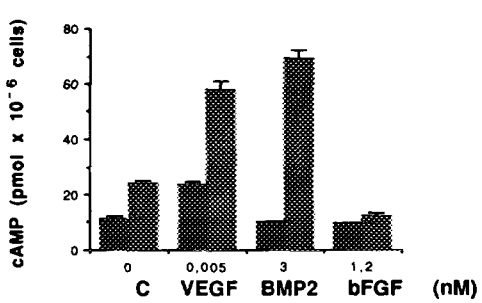


Figure 5. cAMP production by OB cells exposed 6 days to 0.005 nM VAS/VEGF nM, 3 nM BMP2, 1.2 nM bFGF in absence (■) or presence (■) of 400 ng/ml of PTH 1-34. Data are the means +/- SEM of triplicate dishes and are representative of 5 experiments.

single high affinity site, by contrast to its binding to vascular endothelial cells (12,15). The recent cloning of 2 type III tyrosine kinases, KDR (16) and flt-1 (17) encoding VAS/VEGF receptors has not yet disclosed whether the biological activities that they transduced are redundant or not. Our assays to detect the presence of the corresponding mRNAs in reverse transcribed cDNA subjected to polymerase chain reaction using primers designed on the published human sequences, although they allowed the detection of these mRNA in human umbilical vein endothelial cells, have been so far unsuccessfull. Since the bovine sequences are not yet published it remains questionable whether OB express or not the bovine equivalent of KDR or flt-1 or a still unknown receptor. Moreover it has not yet been reported if the activation of these receptors mediates or not an increase of cAMP as in occurs in OB cells.

OB cells did not proliferate but migrated actively under VAS/VEGF addition as do other cells which bind this growth factor on an apparent single site such as corneal endothelial cells, lens epithelial cells (8) or monocytes (9). To further characterize the action of VAS/VEGF on OB cells we compared it to bFGF, the prototype of the angiogenic growth factors and to BMP-2, the prototype of the bone growth factors, on the modulations of 2 biochemical markers of osteoblastic differentiation. Alkaline phosphatase expression have been shown to occur on growth arrested osteoblasts (13,18) and if it is not induced by bFGF, which is a potent osteoblast mitogen (11), it is induced by BMP-2 and VAS/VEGF. Adenylate cyclase activity modulation by PTH 1-34 which is a biochemical marker of the mature osteoblastic phenotype was also increased by both growth factors.

The action of VAS/VEGF on OB cells was detected in all the bioassays tested at doses similar to that required when vascular endothelial cells are used as target cells. However if the maximal effects obtained upon BMP-2 or VAS/VEGF stimulation of OB cells were similar, the optimal doses of VAS/VEGF were 100 fold lower than that of BMP-2. Further experiments would be needed to determine if VAS/VEGF is more potent that BMP-2 in other bioassaays including in vivo experiments such as fracture repair.

In conclusion, these data strengthen the paradigm that VAS/VEGF activity is not restricted to vascular endothelial cells and that it could modulate osteoblastic differentiation.

ACKNOWLEDGMENTS

We would like to thank Drs. Wang and Prats for the generous gifts of recombinant BMP-2 and bFGF, respectively. This work was supported by the Centre National de la Recherche Scientifique (ATIPE N°3). VM is a recipient of a CIFRE-CNRS fellowship.

REFERENCES

1. Plouët, J., Schilling, J., and Gospodarowicz, D. (1989) EMBO J. 8, 3801-3806.
2. Ferrara, N., and Henzel, W.J. (1989) Biochem. Bioph. Res. Commun. 161, 851-856.
3. Connolly, D.T., Olander, J.V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B.L., Leimgruber, R., and Feder, J. (1989) J. Biol. Chem. 264, 20017-20024.
4. Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.W., and Ferrara, N.(1989) Science. 246, 1306-1309.
5. Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D.T. (1989) Science. 246, 1309-1312.
6. Tischer, E., Gospodarowicz, D., Mitchel, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J.C., and Abraham, J.A. (1989) Biochem. Bioph. Res Commun. 165, 1198-1206.
7. Praloran, V., Mirshahi, S., Favard,C., Moukadiri, H., and Plouët, J. (1991) C.R.A.S. III, 21-26.
8. Moukadiri, H., Favard, C., Bikfalvi, A., and Plouët, J. (1991) In Lenfant, C., Paoletti, R., Albertini, A. (eds) : "Symposium on Biotechnology of Growth factors" Karger, pp. 123-128.
9. Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familletti, P.C., Pan, Y.C.E., Olander, J.V., Connolly, D.T.,and Stern, D. (1990) J. Expl. Med. 172, 1535-1546.
10. Fiebich, B., Jager, B., Schollmann, C., Weindel, K., Wilting, J., Kochs, G., Marme, D., Hug, H.,and Weich, H.A. (1993) Eur. J. Biochem. 211, 19-26.
11. Globus, R.K., Plouët, J., and Gospodarowicz, D. (1989) Endocrinology. 124, 1539-1547.
12. Plouët, J., and Moukadiri, H., (1990) J. Biol. Chem. 265, 22071-22074.
13. Sodek, J., and Berkman, F.A. (1987) Methods in Enzymology. 145, 303-323.
14. Wray, H.L., and Glinos, A.D. (1978) Am. J. Physiol., 234, 431-438.

15. Vaisman, N., Gospodarowicz, D., and Neufeld, G.J. (1990) Biol. Chem. 265, 19461-19466.
16. Terman, B.I., Vermazen, M.D., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D., and Böhlen, P. (1992) Biochem. Biophys. Res. Commun. 34, 1578-1586.
17. De Vries, C., Escobedo, J., Ueno, H., Houck, K., Ferrara, N., and Lewis, L.T. (1992) Science. 255, 989-991.
18. Takuwa, Y., Ohse, C., Wang, E.A., Wozney, J.M., and Yamashita, K. (1991) Biochem. Biophys. Res. Commun. 174, 96-101.