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Growth inhibitory activity of atrial natriuretic factor in rat glomerular mesangial cells

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Atrial natriuretic factor (ANF) binding sites, or ANF-induced cGMP accumulation, have been shown in numerous tissues that may not be intimately involved in volume homeostasis. In a series of experiments, we found that quiescent rat glomerular mesangial cells in culture could be reactivated to enter the cell cycle, and that ANF had potent inhibitory effects on proliferation of these cells. [^{3}H]Thymidine incorporation increased by fivefold in reactivated compared to quiescent cells, and ANF in physiologic concentrations inhibited this incorporation by 48%. Thus, ANF appears to be a multifunctional peptide, consistent with a number of peptide growth factors.

Atrial natriuretic factor; Growth inhibitor; Growth factor; DNA synthesis; [^{3}H]Thymidine; (Mesangial cell)

1. INTRODUCTION

The majority of work published on atrial natriuretic factor (ANF) deals with the role of the hormone in volume homeostasis. We have been impressed with the fact that ANF binding sites, or ANF-induced cGMP accumulation, have been shown in numerous tissues that may not be intimately involved in volume homeostasis. For example, ANF binding sites exist in cultured rat lung fibroblasts [1], and ANF stimulates cGMP accumulation in 3T3 fibroblasts [2] as well as homogenates of testis, intestine, lung and liver [3]. In a series of experiments, we have shown that quiescent rat glomerular mesangial cells in culture can be reactivated to enter the cell cycle, and that ANF has potent inhibitory effects on proliferation of these cells. These findings suggest that ANF should be considered a circulating hormone, and possibly an autacoid, with diverse effects on diverse tissues.

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2. MATERIALS AND METHODS

RPMI 1640 was purchased from Hazleton, Lenexa, KS. Fetal bovine serum was from HyClone, Logan, UT. Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DME/F12) was from Sigma, St. Louis, MO. [^{3}H]Thymidine (spec. act. 6.7 Ci/mmol) was from New England Nuclear, Boston, MA. Insulin, transferrin, selenium, and fibroblast growth factor (FGF) were from Collaborative Research, Bedford, MA. Bovine serum albumin and soybean lipids were from Boehringer Mannheim, Indianapolis, IN. Atriopeptin III (APIII) was purchased from Bachem, Torrance, CA.

Glomeruli were isolated from the kidneys of 100 g male Sprague-Dawley rats as previously described [4]. Primary glomerular mesangial cell strains were started from isolated glomeruli using the method of Lovett et al. [5]. Cells were grown in RPMI 1640 media containing 17% fetal bovine serum. Monolayers from passages 1–4 were held in serum-free DME/F12 for 4 days since preliminary work had indicated that by three to four days, the cells incorporate a minimum amount of [^{3}H]thymidine, indicating a quiescent state. A defined mesangial medium (DMM) including DME/F12 plus 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml selenium, 1 mg/ml bovine serum albumin, and 50 μg/ml soybean lipids was utilized to reactivate the mesangial cells. Monolayers were exposed to DMM plus agonists as described, plus 1 μCi/ml [^{3}H]thymidine for 48 h. The reaction was terminated, and DNA was extracted to measure [^{3}H]thymidine incorporation by a slight modification of a previously described technique [6]. Briefly, the cells were washed to remove free [^{3}H]thymidine, lysed and solubilized in SDS, and exposed to ice-cold 10%

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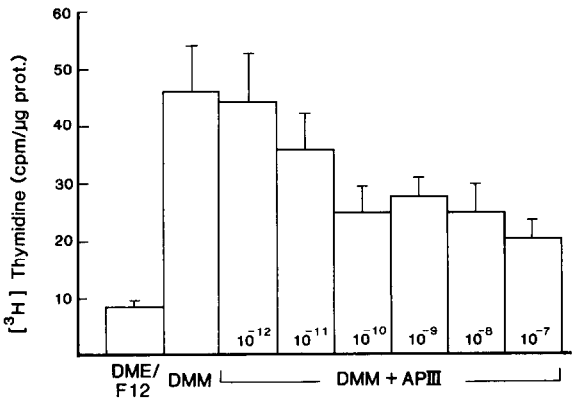

Fig.1. [^{3}H]Thymidine incorporation into DNA of cultured rat glomerular mesangial cells. Results represent mean ± SE of triplicate determinations from 4–8 different cell strains. Numbers given in bars represent APIII concentration (M). Abbreviations as described in text. $p < 0.05$, DMM vs DME/F12, independent t-test. $p < 0.05$, DMM + APIII (10^{-10} M–10^{-7} M) vs DMM, independent t-test.

trichloroacetic acid (TCA). The particulate fraction containing DNA-associated [^{3}H]thymidine was separated from free cytosolic counts by depositing the contents of each well onto glass microfibre filters, and washing with TCA. The filters were dried and counted. Protein was measured in representative wells by the Lowry method [7].

Results are expressed as means ± SE. Statistical significance was determined by use of Student's t-test for unpaired data. Data were considered statistically significant if $p < 0.05$.

3. RESULTS

Fig.1 demonstrates that [^{3}H]thymidine incorporation into quiescent cells in DME/F12 was low at 8.5 ± 1.1 cpm/μg protein. Reactivation of the cells by DMM increased [^{3}H]thymidine incorporation significantly to 46.0 ± 8.4 cpm/μg protein, representing a greater than 5-fold increase. Addition of the synthetic ANF peptide APIII to the DMM in concentrations from 10^{-12} to 10^{-7} M significantly inhibited [^{3}H]thymidine incorporation into mesangial cells. A half-maximal response was seen at approximately 10^{-11} M, representing a concentration in the physiologic range. Additionally, the maximal inhibitory effect, seen at concentrations of APIII 10^{-10} M and higher, was quite potent, averaging 48% of the total DMM-stimulated activity.

Table 1 extends these findings. In cultured mesangial cells exposed to the growth factor FGF plus DMM, [^{3}H]thymidine incorporation increased 7-fold above quiescent values (DME/F12), and 30% above DMM alone. Addition of APIII significantly inhibited [^{3}H]thymidine incorporation in the DMM plus FGF-stimulated cells by an average of 49%. In summary, addition of APIII inhibited [^{3}H]thymidine incorporation by approximately 50% in mesangial cells incubated in DMM or DMM plus FGF. The added proliferative effect of DMM plus FGF over DMM alone was blocked by 50% by APIII. Therefore, these experiments indicate a lack of specificity to the inhibitory effect of APIII on mesangial cell growth and suggest an undefined common mechanism of action.

Table 1

[^{3}H]Thymidine (cpm/μg protein)

Observation	DME/F12	DMM	DMM + APIII	DMM + FGF	DMM + FGF + APIII
1	3.5	48.0	26.7	55.2	27.0
2	8.8	38.0	21.0	44.9	22.5
3	7.8	34.3	15.8	40.3	20.6
4	7.4	44.8	20.8	56.3	29.3
5	6.6	34.7	18.1	57.6	30.5
6	8.0	35.2	18.8	50.4	27.2
Mean ± SE	7.0 ± 0.8	39.2 ± 2.4[a]	20.2 ± 1.5[b]	50.8 ± 2.8[c]	26.2 ± 1.6[d]

[a] $p < 0.05$ vs DME/F12, independent t-test
[b] $p < 0.05$ vs DMM, independent t-test
[c] $p < 0.05$ vs DMM, independent t-test
[d] $p < 0.05$ vs DMM + FGF, independent t-test

[^{3}H]Thymidine incorporation into DNA of cultured rat glomerular mesangial cells. Results represent observations from 2 separate cell strains. FGF concentration 100 ng/ml. APIII concentration 10^{-7} M. Abbreviations as described in text

Table 2

[^{3}H]Thymidine (cpm/well)

Condition	Total cell-associated	DNA-associated
DME/F12	1168 ± 189	254 ± 41
DMM	5454 ± 645	1558 ± 184
DMM + APIII (10^{-8} M)	5966 ± 606	810 ± 93

Results represent the mean ± SE of triplicate determinations from 3 separate experiments. DNA-associated counts determined as described in section 2. Total cell-associated counts determined by counting entire solubilized cell fraction. Abbreviations as described in text

It was important to show that inhibitory effects of ANF on [^{3}H]thymidine incorporation truly reflect inhibition of DNA synthesis and not simply inhibition of active transport of the thymidine through the cell membrane [8]. Therefore, experiments were designed to measure not only DNA-associated [^{3}H]thymidine, but also total cell-associated [^{3}H]thymidine. Table 2 summarizes these data. As expected, there was an excess of total cell-associated versus DNA-associated [^{3}H]thymidine. The important finding, however, was that in the presence of DMM plus APIII, there was no reduction in total counts compared to DMM alone. Only DNA counts fell. This argues against an inhibitory effect of ANF on active transport of thymidine into the cell, and argues for an inhibitory effect of ANF on DNA synthesis.

4. DISCUSION

The results described in this report demonstrate the novel finding that ANF is an inhibitor of mesangial cell proliferation. These cells are of interest in this regard since they are involved in proliferative glomerular diseases, and also have properties in common with vascular smooth muscle cells as well as macrophages [9]. In addition, the mesangial cell is clearly a target site for ANF [4,10]. An extensive review of the literature revealed very little on the subject of ANF and cell growth. A 1986 study revealed that ANF suppressed basal and growth hormone-releasing factor-stimulated secretion of growth hormone from anterior lobe cells of rat pituitary [11]. These findings could suggest an indirect role for ANF in cell growth via the regulation of growth hormone secretion. ANF was found to stimulate [^{3}H]thymidine incorporation into the DNA of bovine adrenal glomerulosa cells in culture [12], suggesting a growth-stimulating effect of ANF on adrenal zona glomerulosa cells. Contrary to these findings, an in vivo infusion of ANF induced atrophy of zona glomerulosa cells of rat adrenals [13], indicating that ANF exerted inhibitory effects on growth of rat zona glomerulosa. It will be important to document whether the growth inhibitory properties of ANF are widely distributed in mammalian cells.

The present work does not clarify the cellular mechanisms by which ANF inhibits growth. One might speculate that previously defined effects of ANF on cellular physiology may be involved. For example, ANF has been shown to stimulate cGMP accumulation [4] and lower free cytosolic calcium [10] in mesangial cells. It is therefore of interest that cGMP may modify cell proliferation [14], and that various growth factors cause an elevation of cytosolic free calcium [15].

The natriuretic, vasorelaxant, and growth-inhibitory effects of ANF indicate that ANF is a multifunctional peptide, consistent with a number of peptide growth factors [16]. There is an increasing body of information relating growth promoters and growth inhibitors to inflammatory and proliferative diseases. For example, the ability of platelet derived growth factor to stimulate migration and proliferation of smooth muscle cells in culture has suggested that this growth factor may be important in the process of atherosclerosis [17]. In addition, loss of responsiveness to or failure to produce growth inhibitors such as transforming growth factor-β may contribute to cancer development [18]. Therefore, the present work may have relevance to pathophysiologic processes involving cellular proliferation and inflammation.

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REFERENCES

[1] Leitman, D.C., Agnost, V.L., Tuan, J.J., Andresen, J.W. and Murad, F. (1987) Biochem. J. 244, 69–74.
[2] Appel, R.G. and Dunn, M.J. (1985) Trans. Assoc. Am. Phys. 98, 85–91.

[3] Waldman, S.A., Rapoport, R.M. and Murad, F. (1984) J. Biol. Chem. 259, 14332–14334.
[4] Appel, R.G., Wang, J., Simonson, M.S. and Dunn, M.J. (1986) Am. J. Physiol. 251, F1036–F1042.
[5] Lovett, D.H., Sterzel, R.B., Kashgarian, M. and Ryan, J.L. (1983) Kidney Int. 23, 342–349.
[6] Arnqvist, H.J., Ballermann, B.J. and King, G.L. (1988) Am. J. Physiol. 254, C411–C416.
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[8] Grunicke, H., Hirsch, F., Wolf, H., Bauer, U. and Kiefer, G. (1975) Exp. Cell Res. 90, 357–364.
[9] Striker, G.E. and Striker, L.J. (1985) Lab. Invest. 53, 122–131.
[10] Appel, R.G., Dubyak, G.R. and Dunn, M.J. (1987) FEBS Lett. 224, 396–400.
[11] Shibasaki, T., Naruse, M., Yamauchi, N., Masuda, A., Imaki, T., Naruse, K., Demura, H., Ling, N., Inagami, T. and Shizume, K. (1986) Biochem. Biophys. Res. Commun. 135, 1035–1041.
[12] Horiba, N., Normura, K., Saito, Y., Demura, H. and Shizume, K. (1985) Biochem. Biophys. Res. Commun. 132, 261–263.
[13] Mazzocchi, G., Rebuffat, P. and Nussdorfer, G.G. (1987) J. Steroid Biochem. 28, 643–646.
[14] Maca, R.D. (1984) Immunopharmacology 8, 53–60.
[15] Moolenaar, W.H., Tertoolen, L.G.J. and De Laat, S.W. (1984) J. Biol. Chem. 259, 8066–8069.
[16] Sporn, M.B. and Roberts, A.B. (1988) Nature 332, 217–219.
[17] Antoniades, H.N. and Owen, A.J. (1982) Annu. Rev. Med. 33, 445–463.
[18] Marx, J.L. (1988) Science 239, 975–976.