Influence of Ruminant Amniotic Fluid Fractions on Fibroblast and Lymphocyte Proliferation

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Abstract

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Different substances of amniotic fluid influence the cell proliferation and differentiation of developing animal fetus. The aim of this study was to determine the mitogenic effect of some peptide components of bovine amniotic fluid on bovine peripheral blood lymphocytes using methyl tetrazolium (MTT) colorimetric assay. The next aim of our work was to determine the mitogenic activity of ovine amniotic fluid fractions of peptide nature on benzo-α-pyrene transformed BALB/c 3T3 mouse fibroblasts (BP-A31 cells) by use of ^{3}H-thymidine incorporation into nucleus DNA and in conclusion, to compare the mitogenic activity of ruminant amniotic fluid fractions on different indicator cells. According to our study, inhibiting effect was found only in the case of separated bovine amniotic fluid (Peak I) and ovine amniotic fluid (B fraction). On the other hand, we have observed activation of lymphocytes by other fraction of bovine amniotic fluid (Peak II) and also of BP- A31 cells by fraction A in case of ovine amniotic fluid. The proliferation of peripheral lymphocytes was not significantly changed after the addition of natural bovine amniotic fluid likewise when the delipidated ovine amniotic fluid was added to BP-A31 cells, there was no effect on ^{3}H-thymidine incorporation. Our results suggest that with testing the proliferation effect, the selection of indicator cells is of great importance since various cell types respond in different ways to the same substances.

Bovine amniotic fluid, ovine amniotic fluid, BP-A31 cells, peripheral blood lymphocytes, mitogenic activity, inhibition of cell proliferation

Amniotic fluid is rich in protein substances (hormones, cytokines, polypeptide growth factors), which influence cell proliferation and differentiation of developing foetus (Parvin et al. 1994; Tamatani et al. 1988). The immunomodulatory properties of amniotic fluid have been extensively demonstrated in humans, mice and rats (Yoshimura et al. 1991), and suggest that the regulatory activity of amniotic fluid may be crucial in regulating maternal immunity during pregnancy as well as neonatal responsiveness in the foetal and neonatal periods (Parvin et al. 1994). According to some observations the amniotic fluid plays an important role in the protection of foetuses from bacterial infections (Saito et al. 1993). In the contrary other investigators have found that human amniotic fluid (Lang et al. 1994), murine and rat amniotic fluid (Parvin et al. 1994) suppress mitogen-stimulated lymphocyte proliferation. It is known that certain functions of the immune system can be modulated by insulin-like growth factor-I and -II (IGF-I and IGF-II) present in amniotic fluid (Kooijman et al. 1992).

The purpose of this study was to estimate mitogenic activity of peptide substances present in ruminant amniotic fluid obtained by separation on Sephadex G-10. Bovine peripheral blood lymphocytes and benzo-α-pyrene transformed BALB/c 3T3 mouse fibroblasts (BP-A31 cell line) were used as indicator cells.

Materials and Methods

Collection and fractionation of amniotic fluid

Amniotic fluid was obtained by laparotomy, hysterotomy and puncture of amniotic sac of sheep in 10th week of pregnancy and cows in 16th week of pregnancy (species differences can be neglected because the stages of developing

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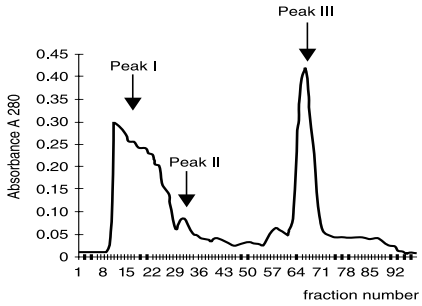


Fig. 1. Gel chromatography of bovine amniotic fluid on Sephadex G-10 (2×45 cm), elution in 0.04 mol.l^{-1} NH_4HCO_3 at a flow rate of 9.5 ml.h^{-1}. The content of peptides was determined by measuring the absorbance at wave-length of 280 nm.

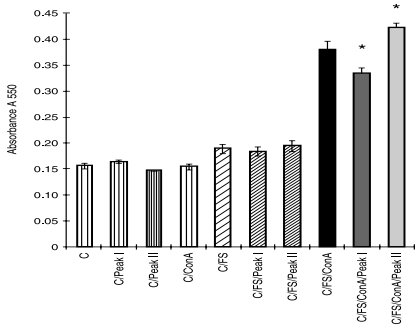
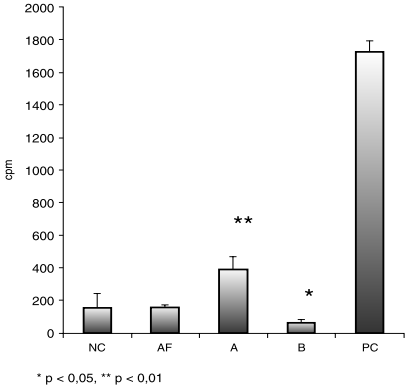


Fig. 3. Proliferation of peripheral blood lymphocytes in the presence of ConA and fractions obtained by gel filtration (Peak I and Peak II). Results are shown as means of three replicates. C- cells in complete medium, C/ConA, C/Peak I and C/Peak II - cells in complete medium supplemented with 10 µg. ml^{-1} of ConA, Peak I or Peak II, C/FS - cells in the complete medium supplemented with 10% foetal calf serum, C/FS/ConA, C/FS/Peak I and C/FS/Peak II - cells in the complete medium supplemented with of foetal calf serum and ConA, Peak I or Peak II, C/FS/ConA/Peak I or C/FS/ConA/Peak II - cells in complete medium supplemented with foetal calf serum, ConA, Peak I or Peak II.



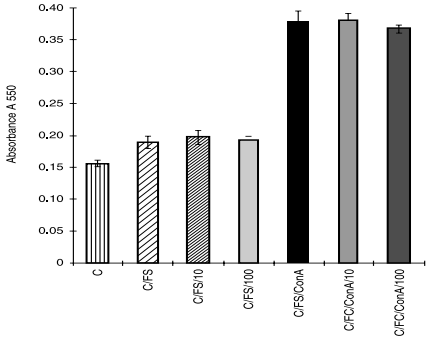


Fig. 2. Proliferation of peripheral blood lymphocytes in the presence of natural bovine amniotic fluid. Results are shown as the means of three replicates. C - cells in complete medium, C/FS - cells in the complete medium supplemented with 10% of foetal calf serum, C/FS/ConA - cells in the complete medium supplemented with 10% of foetal calf serum and 10 µg. ml^{-1} of ConA, C/FS/10 or C/FS/100 - cells in complete medium supplemented with foetal calf serum and natural amniotic fluid diluted 10 times or 100 times, C/FS/ConA/10 or C/FS/ConA/100 - cells in complete medium supplemented with foetal calf serum, ConA and natural amniotic fluid diluted 10 times or 100 times.

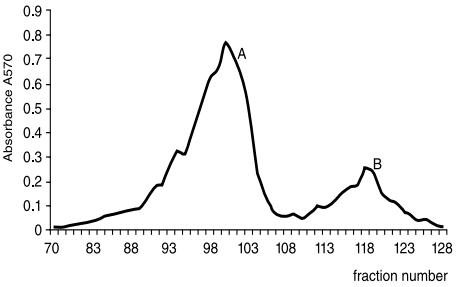


Fig. 4. Gel chromatography of ovine amniotic fluid on Sephadex G-10 column (5 × 70 cm), elution in 0.04 mol.l^{-1} NH_4HCO_3 at a flow rate of 12 ml.h^{-1}. Fractions were determined by ninhydrin reaction (A 570).

Fig. 5. Mitogenic activity of ovine amniotic fluid. NC - negative control (cells in complete medium), AF - non-separated ovine amniotic fluid, A, B - fractions obtained by gel chromatography on Sephadex G-10, PC - positive control (cells in complete medium supplemented with 6% foetal calf serum).

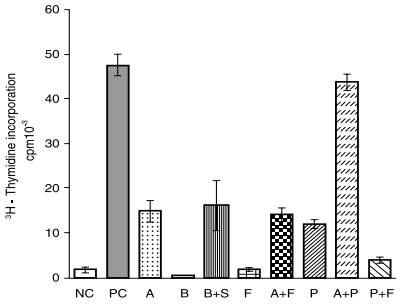


Fig. 6. Effect of Sephadex G-10 column fractions (the final concentrations were 25 μg.ml^{-1}) on ^{3}H-thymidine incorporation by BP-A31 cells. At the same time, the effect of tetradecanoyl phorbolacetate (TPA) was determined, alone and in combination with fraction A. The inhibition of the mitogenic effects of TPA (100 ng.ml^{-1}) and of fraction A by forskolin (10^{-5} mol.l^{-1}) were also assayed. Foetal bovine serum (6%) was used as positive control and serum-free MEMα as negative control. Results are expressed as means ± SD of triplicate cultures. The experiment was repeated twice with similar results. NC - negative control, PC - positive control, A,B - fractions A, B, F- forskolin, P- TPA.

ovine and bovine foetuses are approximately the same). The samples were delipidated with chloroform and stored in a lyophilized form. Diluted sample was loaded into a Sephadex G-10 column (2 × 45 cm for bovine and 5 × 70 cm for ovine amniotic fluid). Elution was performed using 0.04 mol.l^{-1} NH_4HCO_3 at a flow rate 9.5 ml·h^{-1} (bovine amniotic fluid) and 12 ml·h^{-1} (ovine amniotic fluid). The content of peptides was determined by measuring of the absorbance at 280 nm and by ninhydrin reaction. Natural amniotic fluid of cow nature - diluted 10 times and 100 times with complete culture medium RPMI-1640, and fractions of the first and second peak were tested for mitogenic activity.

Mitogen - induced proliferation of peripheral blood lymphocytes

Bovine peripheral blood lymphocytes were obtained by centrifugation using Histopaque-1077. Isolated lymphocytes (3.5 × 10^6 cells.ml^{-1}, in triplicates) suspended in the complete culture medium (RPMI-1640 with 100 units of penicillin and 100 μg of streptomycin per 1 ml plus 2 mmol·l^{-1} of glutamine, without phenol red) were added into 24-well flat-bottom plastic plates in a final volume of 1 ml. Some wells were supplemented with 10% of foetal calf serum (FS). Concanavalin A (Con A) and fractions of amniotic fluid after gel filtration (Peak I, Peak II) were diluted in the complete culture medium and added into the wells at a final concentration of 10 μg·ml^{-1}. Mitogenic activity of the amniotic fluid (10 μl) and its fractions was estimated using optimised MTT colorimetric assay described by Blahovec and Sobeková (2001). Determination was made three times in triplicates.

Fibroblast proliferation

Mouse BP-A31 fibroblasts were maintained in minimal essential medium modification α (MEMα) supplemented with 6% foetal calf serum (FS) in a humidified air/CO_2 (5%) atmosphere. For the detection of mitogenic activity, 20 × 10^3 cells were seeded per well in 24 flat bottom well boxes. After 24 h, the medium was replaced with 1 ml MEMα plus 2.5 μmol.l^{-1} $FeSO_4$ and the cells were allowed to enter quiescence for additional 24 h. ^{3}H-thymidine (2μCi) was added to the wells, together with column fractions to be tested. After 24 h, the incorporation was terminated by acidification with 1 mol·l^{-1} ascorbic acid. The cells were fixed and washed with 5% trichloroacetic acid and solubilized in 0.1 mol·l^{-1} NaOH for counting in a liquid scintillation spectrometer. Forskolin (10^{-5} mol·l^{-1}) and tetradecanoyl phorbolacetate (100 ng·ml^{-1}) were used to investigate the role of protein kinase A and protein kinase C signalling pathways.

Statistical analysis

The results are given as means ± SD. Statistical significance of differences between the obtained results was evaluated by the Student's *t*-test.

Results

Bovine amniotic fluid was fractionated by gel chromatography into three peaks (Fig. 1). Non-fractionated amniotic fluid and peptides from Peak I and Peak II were tested for mitogenic activity. Fig. 2 shows that ConA-induced proliferation of peripheral lymphocytes was not significantly changed after the addition of natural bovine amniotic fluid. However, fractions of amniotic fluid obtained by gel chromatography showed significantly different activity (Fig. 3). Inhibiting effect was found in the case of separated amniotic fluid (Peak I). On the other hand, we have observed activation of lymphocytes by other fraction of amniotic fluid (Peak II). Peak III had no effect (data not shown).

When the delipidated ovine amniotic fluid was added to BP-A31 cells, there was no effect on ^{3}H-thymidine incorporation. However, after gel filtration on Sephadex G-10 (Fig. 4), the fraction eluted in the void volume (fraction A) was able to initiate the cell cycle in BP-A31 cells, in contrast, fraction eluted in the total volume of the column (fraction B) inhibited the cell cycle (Fig. 5).

In order to obtain information concerning the functional characteristics of the growth factor(s) responsible for the observed mitogenic effect of fraction A, we tested the activity of these fractions in the presence of tetradecanoyl phorbolacetate (activator of protein kinase C) or forskolin (activator of adenylate cyclase). Forskolin did not alter the mitogenic activity of fraction A, whereas it abolished the effect of TPA (Fig. 6). The activities of fraction A and TPA were approximately additive.

Discussion

During the rapid development of the embryo and foetus peptide growth factors are associated with tissue induction, the clonal growth of stem-cell populations, and the appearance of differentiated function in the presumptive organs. Insulin-like growth factor-I and -II (IGF-I and -II) are the polypeptide growth factors that are potent mitogens for cells derived from all primitive germ layers. The IGFs appear early in embryogenesis during and after the condensation of presumptive organs and tissues and they are involved in growth and differentiation of dog dental pulp cells (Onishi et al. 1999), in proliferation and differentiation of myoblasts, chondrocytes and hematopoetic cells (Zumkeller 2002) and brain cell types (Hill 1989).

It is known that amniotic fluid contains insulin-like growth factors (IGF-I and -II) and their precursor forms (fraction A) besides other growth factors (Blahovec et al. 2001). IGF-I is associated to specific binding protein, IGFBP-1 (Nonoshita et al. 1994). This binary complex not only prolongs biological half-life of IGF-I and transports it to the target cells, but also decreases or eliminates its biological activity (Liu et al. 1991). In our experiment, non-fractionated ovine amniotic fluid had no effect on proliferation of BP-A31 cells. However, after gel chromatography, peptide molecules of fraction A caused initiation of BP-A31 cell division. This mitogenic activity displayed the characteristics of IGF because it was not inhibited by forskolin (inducer of adenylate cyclase) and it was additive with activity caused by phorbolesters. The void volume fraction A contains all molecules with $M_r \geq 700$ Da, it means that also IGF-I and -II (peptide molecules with molecular weight of 7.6 kDa and 7.4 kDa, respectively), precursor forms of IGF-II (17 - 30 kDa) and IGFBPs (40 - 45 kDa) are present there (Blahovec et al. 2001). Fraction B contains small molecules with $M_r \leq 700$ Da and these peptides inhibited the proliferation of chemically transformed mouse fibroblasts, but did not influence the proliferation of carcinoma cell line MCF_7 (data not shown).

Suppression of the ConA-induced proliferation of splenic lymphocytes and thymocytes has been shown with murine amniotic fluid (Parvin et al. 1994). Down-modulatory activity has been found also for human, murine and rat amniotic fluid when the peripheral mononuclear cells were used as target cells (Lang et al. 1994; Parvin et al. 1994). Normal freshly collected human lymphocytes expressed mRNAs for both IGF-I receptor and IGF-II receptor but no expression of the corresponding growth factors was detectable. After stimulation with phytohaemagglutinin the lymphocytes, however, expressed both IGF-I and IGF-II (Nyman and Pekonen 1993). IGF- I and IGF-II also augmented the lectin-induced proliferation of purified T lymphocytes (Kooijman et al. 1992). In our work, ConA - induced proliferation of peripheral lymphocytes was inhibited by substances present in Peak I of separated bovine amniotic fluid and activated by smaller peptide molecules (Peak II).

Our results are interesting because we have obtained one fraction of amniotic fluid with stimulatory properties and another one with inhibitory effects on fibroblasts but their effects are absolutely opposite in case of lymphocytes used as indicator cells. It is known that the same growth factor can act differently on the different cell types, can act differently at different concentrations or at different times on the same cell type (McKay 1993).

Vplyv frakcií amniónovej tekutiny prežúvavcov na proliferáciu fibroblastov a lymfocytov

Proliferácia a diferenciácia buniek vyvíjajúceho sa plodu je ovplyvňovaná rozličnými látkami prítomnými v amniónovej tekutine. Cieľom našej práce bolo stanoviť mitogénny účinok niektorých látok peptidovej povahy z bovinnej amniónovej tekutiny na periférne lymfocyty kolorimetrickým metyltetrazóliovým testom a stanovenie mitogénnej aktivity peptidových frakcií ovčej amniónovej tekutiny na chemicky transformované BALB/c 3T3 myšie fibroblasty (bunky BP-A31) meraním rádioaktivity ^{3}H-tymidínu inkorporovaného do DNA buniek. Na základe týchto meraní sme porovnávali mitogénnu aktivitu frakcií amniónovej tekutiny prežúvavcov v rovnakom štádiu gravidity na rôzne typy buniek. Z výsledkov našej práce vyplýva, že inhibičný účinok bol dokázaný u frakcií píku I bovinnej amniónovej tekutiny a frakcie B ovčej amniónovej tekutiny. Na druhej strane bola pozorovaná aktivácia lymfocytov frakciami píku II a proliferácia buniek BP-A31 frakciou A ovčej amniónovej tekutiny. Proliferácia periférnych lymfocytov nebola ovplyvnená prídavkom prirodzenej hovädzej amniónovej tekutiny podobne ako keď delipidovaná ovčia amniónová tekutina suplementáciou k bunkám BP-A31 nevyvolala inkorporáciu ^{3}H-tymidínu do DNA indikátorových buniek. Na základe našich výsledkov možno konštatovať, že pri testovaní proliferačného účinku je veľmi dôležitý výber indikátorových buniek, nakoľko rôzne typy buniek reagujú na rovnaké látky odlišným spôsobom.

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