α_1 -Acid Glycoprotein in Bovine Lymphocytes

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ABSTRACT. α_1 -acid glycoprotein (α_1 AGP) in bovine lymphocytes was detected by indirect immunofluorescence antibody test on approximately 90% of peripheral blood lymphocytes as well as lymphocytes in lymph nodes and spleens. α_1 AGP isolated from bovine lymphocytes revealed the consequent 5 spots in an acid portion (pI 3.5–4.5) with molecular weight (M. W.) 27,000 to 39,000 daltons on two dimensional gel electrophoresis. α_1 AGP obtained from the supernatant of lymphocytes cultured with bovine interleukin 2 (IL-2) showed the consequent 3 spots with pI 3.5–4.0 and M. W. 29,000–36,000 daltons. The α_1 AGP concentration in non-stimulated lymphocyte was found to be 21 ng/10⁶ cells by radioimmunoassay. The α_1 AGP levels in lymphocytes cultured with IL-2, concanavaline A, and pokeweed mitogen were increased from the level in non-stimulated cells to 78, 58, 69 ng/10⁶ cells on day 4, respectively. Furthermore, the α_1 AGP level was as high as 90 ng/10⁶ cells in lymphocytes cultured with IL-2 for 35 days. α_1 AGP was detected in culture supernatants of lymphocytes with IL-2 and not with above mentioned mitogens. From these results, bovine α_1 AGP was confirmed to be synthesized in lymphocytes, and much more actively produced in lymphocytes stimulated with IL-2.—**KEY WORDS:** bovine lymphocyte, α_1 -acid glycoprotein.

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α_1 -acid glycoprotein (α_1 AGP) is well known to be elevated in serum levels during numerous conditions including acute inflammation, neoplasm, pregnancy and severe burns [4, 5, 10, 14, 16]. α_1 AGP was considered to be synthesized for secretion primary in liver. Human α_1 AGP was detected not only in liver parenchymal cells [2, 18], but also in myocardium [19], breast epithelial cells [7], and blood cells as lymphocytes, neutrophils, monocytes, and platelets [1, 6, 12, 20]. It has also been reported that human serum α_1 AGP was secreted from lymphocytes and markedly increased in inflammatory and lymphoproliferative diseases. These findings suggested the possibility of α_1 AGP synthesis in lymphocytes and of its increase in secretion [3, 6, 17] associated with these diseases. However, few information concerning synthesis of α_1 AGP in bovine cells except liver parenchymal cells has been available.

Therefore, α_1 AGP synthesis in bovine lymphocytes was investigated.

MATERIALS AND METHODS

Lymphocyte: Peripheral blood lymphocytes (PBL) were obtained from heparinized blood of cattle. Enriched lymphocyte fractions were separated with lymphoprep (Daiichi Kagaku, Japan). For the cultivation of lymphocytes, crude interleukin 2 (IL-2) was prepared from concanavalin A (ConA, Sigma, U.S.A.)-stimulated PBL from cattle, according to the method of Miller-Edge *et al.* [11].

Lymphocyte culture: PBL were cultivated in RPMI 1640 medium containing 10% rabbit serum at 37°C in a 5% CO₂-humidified atmosphere. The cells were stimulated with bovine IL-2 (10%), ConA (10 μ g/ml), pokeweed mitogen (PWM, 2 μ g/ml), or lipopolysaccharide (LPS, 10 μ g/ml). The

cells were collected and lysed in phosphate buffered saline (PBS, 0.14 M NaCl and 0.01 M phosphate buffer pH 7.2) containing 1% Triton X-100, 1% ethanol, 100 μ g/l soybean trypsin inhibitor, and 10 μ g/l pepstatin A. The lysed cell suspensions were sonicated at 20 kHz for 5 min and centrifuged at 10,000 rpm for 30 min [1, 6].

Anti-bovine α_1 AGP rabbit serum: Previously described techniques were used to obtain α_1 AGP from bovine serum [9]. It was shown using Ouchterlony test or immunoelectrophoresis that the antiserum revealed a single arc against normal bovine serum.

Indirect immunofluorescence test: Lymphocytes were washed and suspended in 100 μ l of PBS added 0.02% sodium azide. They were added with diluted rabbit anti-bovine α_1 AGP serum (100 μ l of 1:8) and incubated at 37°C for 1 hr. Then, the cells were washed three times with PBS and resuspended in 100 μ l of 1:16 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum (Cappel Lab. Inc., USA). After 1 hr at 37°C, the cells were washed and examined under the fluorescence microscope (OPTIPHOTO, Nikon, Japan).

Immunosorbent affinity column chromatography: Immunosorbents were prepared by coupling antibody with CNBr-activated Sepharose 4B Gel (Pharmacia, Sweden). Approximately 3 g freeze-dried powder of the gel was stored in 1 mM HCl for 15 min and washed 3 times with 1 mM HCl on G3-glass filter. Then, the gel was washed with coupling buffer consisting of 0.5 M NaCl and 0.1 M NaHCO₃ (pH 8.3) and mixed with 100 mg of the precipitated protein dissolved in 10 ml of coupling buffer. The gel suspension was gently stirred at 4°C overnight, and the remaining active sites were blocked with 0.2 M glycine (pH 8.0) at room temperature for 2 hr and then washed with coupling buffer and 0.1 M acetate buffer pH 4.0 added with 0.5 M

NaCl by turns. The gel was equilibrated with 0.02 M Tris-HCl buffered saline (TBS, pH 7.4) and packed into the chromatography column (1.0×20 cm).

Samples were dialyzed against TBS on Sephadex G-25 gel filtration and applied to the immunosorbent column. The non-adsorbed protein was washed with the above buffer and then the adsorbed protein was eluted with 0.1 M glycine buffer (pH 2.3). These fractions were collected, dialyzed against PBS, and concentrated by vacuum filtration.

Two dimensional gel electrophoresis: The method by O'Farrell [13] was used with slight modifications. The first dimensional gel was prepared in glass tubing (100×2 mm) containing 1% agarose IEF, 10% Ampholine, and 10% sorbitol. Electrode solution was 0.5 M acetic acid for anode and 0.5 M NaOH for cathode. The gels were run at a constant Watt of 0.4 W/tube (primary current: 1 mA/tube) for 15 min and then at 1 W/tube at 4°C for 30 min. At the end of the electrophoresis, the gels were pushed out and overlaid on the top of SDS-PAGE and run at constant voltage of 80 V for 17 hr. The gels were stained by ultrasensitive silver based color staining reported by Sammons *et al.* [15].

Radioimmunoassay: The 10 μ g of purified serum α_1 AGP was iodinated by the chloramine T method using 0.5 mCi Na¹²⁵I (New England Nuclear, USA) [8]. Fractions were collected after passing through 1×10 cm Sephadex G-25 column made in PBS and counted in a gamma counter (Beckmann, USA). The specific activity on the labeled antigen was 32 μ Ci/g.

The samples or purified α_1 AGP as standard diluted with the same culture medium were incubated with 0.2 ng of ¹²⁵I-labeled α_1 AGP in 50 μ l of buffer, 100 μ l of 1/15 M borate buffer (pH 8.6) containing 0.5% rabbit albumin (Sigma, USA) and 0.01 M EDTA-2Na, and 50 μ l of rabbit anti- α_1 AGP

Fig. 1. Indirect immunofluorescence test for α_1 AGP. A: Lymphocyte. B: Hepatocytes.

Fig. 2. Elution pattern of lymphocytes lysate on immunosorbent affinity column chromatography. Elution with I: 0.02 M tris-HCl buffer (pH 7.5) and II: 0.1 M glycine-HCl buffer (pH 2.3). Column size: 0.8×10 cm. Elution speed: 5 ml/hr.

antiserum ($\times 2000$) at 4°C for 48 hr. Then, 50 μ l of rabbit serum was added as a carrier. After 24 hr at 4°C, 300 μ l of 25% polyethylene glycol 2000 was mixed with precipitated protein and the samples were centrifuged. The pellets were counted for radioactivity. In this method, 0.1 to 1000 ng of α_1 AGP could be assayed.

RESULTS

Immunofluorescence test: A granular membrane pattern of staining was obtained in

approximately 90% of non-fixed PBL, when stained with rabbit anti-bovine α_1 AGP and FITC-conjugated goat anti-rabbit IgG (Fig. 1). The adsorption test showed the decrease or disappearance of the immuno-fluorescence. On frozen sections of spleens and lymph nodes, an effusive fluorescence was shown in cytoplasm of the cells, while a granular fluorescence pattern was detected in cytoplasm of liver parenchymal cells. *Electrophoretic analysis of isolated protein from lymphocytes and lymphocyte culture supernatants:* Elution pattern of lymphocyte

Fig. 3. Immunological analysis of bovine α_1 AGP by Ouchterlony test. S: Bovine whole serum. A: purified bovine α_1 AGP. L: α_1 AGP from lymphocytes. CS: α_1 AGP from supernatants of lymphocytes cultured with IL-2. H: α_1 AGP from bovine hepatocytes. AA: Anti-bovine α_1 AGP antiserum.

Fig. 4. Two dimensional gel electrophoresis of bovine α_1 AGP from lymphocytes (A) and supernatants of lymphocytes cultured with IL-2 (B). Arrow heads show the spots of α_1 AGP with various molecular weights (I: 39,000, II: 36,000, III: 31,000, IV: 29,000, and V: 27,000).

lysate on immunosorbent affinity chromatography is given in Fig. 2. The isolated protein on this column was shown to be precipitated with anti-bovine α_1 AGP by Ouchterlony test (Fig. 3). On two dimensional gel electrophoresis, the protein revealed the consequent 5 spots in an acid

portion (pI 3.5–4.5) with 39,000, 36,000, 31,000, 29,000, and 27,000 daltons (Fig. 4A). The isolated protein from lymphocyte culture supernatants was also precipitated against anti-bovine α_1 AGP antiserum (Fig. 3). On two dimensional gel electrophoresis, the protein revealed the consequent 3 spots

Fig. 5. α_1 AGP concentration in lymphocytes cultured with IL-2 (●—●), Con A (▲—▲), PWM (■—■), and LPS (○—○).

in the most acid portion of pI 3.5–4.0 with 36,000, 31,000, and 29,000 daltons (Fig. 4B).

Concentration of α_1 AGP in lymphocytes: The α_1 AGP concentration in non-stimulated lymphocytes was shown to be 21 ng/ 10^6 cells by radioimmunoassay. The changes of α_1 AGP levels in lymphocytes after cultivation are shown in Fig. 5. The viable cell number of lymphocytes cultured with LPS, Con A, PWM, and IL-2 on day 4 was 6.3×10^5 , 8.0×10^5 , 8.4×10^5 , and 1.6×10^6 cells/ml, respectively. The α_1 AGP concentrations in stimulated lymphocytes with Con A, PWM, or IL-2 were increased on day 4, showing 58, 69, and 78 ng/ 10^6 cells, respectively, but not with LPS. The α_1 AGP level was increased as high as 90 ng/ 10^6 cells in lymphocytes cultured with IL-2 for 35 days.

Concentration of α_1 AGP in lymphocyte culture supernatants: The α_1 AGP concentration in the lymphocyte culture supernatants during cell proliferation is shown in Fig. 6. The α_1 AGP was increased in level when cultured with IL-2, but undetectable in the

Fig. 6. α_1 AGP concentration in culture supernatants of lymphocytes cultured with (●—●) and without (○—○) IL-2.

supernatants of lymphocytes cultured with Con A, PWM, and LPS.

DISCUSSION

A specific immunofluorescence to α_1 AGP was observed on bovine lymphocytes as on liver parenchymal cells, suggesting the presence and synthesis of α_1 AGP in the lymphocytes. Two staining patterns on frozen section, effusive type in lymphocytes and granular type in liver parenchymal cells, might indicate that presenting form and/or distribution pattern differed in these two cells. Gahmberg *et al.* [6] demonstrated that large molecular α_1 AGP with 52,000 daltons, membrane form, was found in normal human T and B lymphocytes, lymphoblasts, granulocytes, and monocytes. These cells were also observed to secrete α_1 AGP with 41,000 daltons as serum form. Furthermore, α_1 AGP in neutrophils was reported to be different in molecular size from that in serum [1]. In this study, bovine α_1 AGP obtained from lymphocytes or lymphocyte culture supernatants showed the 3 or 5 spots with pI 3.5–4.5 and 27,000 to 39,000 daltons of M. W. by two dimensional gel electrophoresis. The two spots with lower molecular weight size (27,000 and 29,000

daltons) were not detected in normal serum or in liver extracts [9]. These 2 spots of α_1 AGP might be originated from lymphocytes.

It was reported that human T or B lymphocytes, when cultured with a stimulator such as mitogen or IL-2, could synthesize α_1 AGP [1, 6]. The concentration of α_1 AGP in bovine lymphocytes increased after cultured with mitogens and IL-2. The synthesis of α_1 AGP in bovine lymphocytes was necessary for the activation of lymphocytes as well as human lymphocytes. The concentration of α_1 AGP could be undetectable by radioimmunoassay in supernatants of lymphocytes cultured with mitogens, but were increased in supernatants of lymphocytes cultured with IL-2 as incubation time passed. The concentration of α_1 AGP in culture supernatants might be dependent on the number of activated cells and/or the subpopulation of lymphocytes stimulated.

From these results, it was indicated that bovine α_1 AGP was also synthesized for secretion in lymphocytes as well as liver parenchymal cells. Its levels were increased associated with lymphocyte activation or stimulation as reported in those of human α_1 AGP[6]. Therefore, the serum concentration of α_1 AGP might be elevated under various conditions as lympho-proliferative diseases, particularly lymphoid cell tumor, by resulting from the enhancement of lymphocyte α_1 AGP synthesis.

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要 約

牛リンパ球における α_1 酸性糖蛋白の産生：岩田祐之・小野憲一郎・長谷川篤彦・友田 勇（東京大学農学部家畜内科学教室）—— α_1 酸性糖蛋白（以下 α_1 AGP）は間接蛍光抗体法で牛リンパ球の約90%に認められた。リンパ球から分離した α_1 AGP は二次元電気泳動法で酸性領域に5つの連続した分画として観察され、その分子量は27,000から39,000 daltons であった。インターロイキン2（以下 IL-2）を添加して培養したリンパ球の培養上清から得た α_1 AGP の分子量は29,000から36,000 daltons であった。ラジオイムノアッセイ法で測定したリンパ球内 α_1 AGP 濃度は、IL-2, Concanavalin A, および Pokeweed mitogen 添加培養時に、無添加の際の 21 ng/10⁶ cells に比べそれぞれ78, 58, 69 ng/10⁶ cells と増加した。とくに、IL-2 添加時の培養35日目には 90 ng/10⁶ cells と著明であった。リンパ球培養上清中の α_1 AGP は IL-2 添加時のみに認められ、その他の mitogen 添加時には測定感度以下であった。以上のことから、 α_1 AGP は牛リンパ球で産生され、さらにその産生はリンパ球が刺激されることにより増加することが判明した。