Modulation of cell surface lectin receptors on K562 human erythroleukemia cells induced by transfection with annexin IV cDNA

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Abstract Annexin IV was found to be highly expressed in various human adenocarcinoma cell lines, but not in an erythroleukemia cell line, K562. We investigated the effects of transfection of human annexin IV cDNA into K562 cells on cell surface lectin receptors. cDNA transfectants were found to be more sensitive to cytotoxic lectins such as *Ricinus communis* agglutinin and wheat germ agglutinin than mock transfectants. The results of flow cytometric analyses with various lectins showed that the transfectants expressed more sugar chains which bind to *Ulex europaeus* agglutinin I and *Maackia amurensis* mitogen than mock transfectants. These results suggest that transfection of annexin IV cDNA increases the expression of α-2,3-sialylated and/or fucosylated sugar chains on the surface.
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Key words: Annexin IV; Lectin; K562; Transfection; Endocytosis

1. Introduction

Annexins are a family of structurally related proteins that bind to phospholipids in a calcium-dependent manner. Annexins are composed of 4 or 8 repeating domains of around 70 amino acids and an N-terminal domain specific for each type. The fact that annexins have no signal peptide confirmed findings that they are abundant in the cytoplasm, and that they are intracellular proteins. However, a number of recent studies have indicated that annexins exist not only in the cytoplasm, but also in the plasma membrane as intrinsic membrane proteins on the cell surface, or that they are extracellularly secreted, depending on the cell type. Although a number of in vitro experiments have indicated that some annexins either exhibit membrane channel activity, inhibit phospholipase A_2 and blood coagulation, transduce mitogenic signals, or are involved in the membrane-cytoskeleton interaction or membrane-related intracellular events such as vesicle transport (reviewed by Raynal and Pollard [1]), their exact biological functions in vivo have not yet been completely elucidated.

The effect of annexin transfection into cells which do not express the corresponding annexin may be useful for elucidating the function of annexins. So far, transfection studies involving the cDNAs of annexins I, II and VI have been reported for some cell lines. The results obtained suggest various properties of annexins, a relationship between annexin I expression and prostaglandin production in arachidonic acid metabolism [2], a relationship between annexin II expression and the expression level or translocation of p11 [3–5], an interaction between annexin II and tissue plasminogen activator [6], a relation between annexin II expression and osteoclast formation or bone resorption [7], and a relationship between annexin VI and cell proliferation or endocytosis of transferrin receptors [8,9].

In this study, we focused especially on the effects on the sensitivities to plant lectins and the cell surface expression of the lectin receptors of the transfection of human annexin IV cDNA into the human erythroleukemia cell line, K562, which expresses no annexin IV mRNA.

2. Materials and methods

2.1. Cell culture

The human erythroleukemia cell line, K562, was donated by the Japan Cancer Research Resource Bank (Tokyo, Japan). K562 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum without antibiotics at 37°C under a humidified atmosphere containing 5% CO_2. At 70% confluency, the cells were passaged by dilution in fresh medium. The cells used in this experiment were free of mycoplasma infection.

2.2. Northern blot analysis

For Northern blot analysis, human annexin IV 2.0 kb cDNA from a human colon carcinoma cell line, a HT29 λgt10 cDNA library [10], was used as a probe. A ^{32}P-labeled probe was generated by random priming using Klenow fragment (Stratagene, La Jolla, CA). Blots were hybridized and washed according to the procedure described by Church and Gilbert [11].

2.3. cDNA transfection and production of stable transfectants

cDNA was ligated into the *Hin*dIII-*Kpn*I site of expression vector pCEP4 (Invitrogen, San Diego, CA). Plasmid DNA was purified using a plasmid DNA purification kit (Qiagen GmbH, Germany). Actively proliferating K562 cells were harvested, washed, and then resuspended in standard phosphate-buffered saline (PBS) at 1×10^7 cells/ml in 0.4 cm cuvettes (Bio-Rad Labs, Hercules, CA) in a total volume of 0.4 ml. Electroporation (Bio-Rad Gene pulser) with purified plasmid DNA (5 μg) was performed at 400 V (250 μF), with a time constant consistently in the range of 5.6–5.9 ms. Transfected cells were selected in

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Abbreviations: PBS, standard phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin; PNA, peanut agglutinin; ABA, *Agaricus bisporus* agglutinin; UEA-I, *Ulex europaeus* agglutinin I; SBA, soybean agglutinin; MAM, *Maackia amurensis* mitogen; SSA, *Sambucus sieboldiana* agglutinin

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medium containing hygromycin B (Calbiochem, San Diego, CA) at a final concentration of 400 μg/ml after 72 h and maintained in the same medium thereafter. After 3 weeks, the cells were cloned by limited dilution. For the selection of cells expressing annexin IV, flow cytometric analysis and immunoprecipitation were performed.

2.4. Preparation of cell lysates, immunoprecipitation, SDS-PAGE and Western blot and lectin blot analyses

Cell lysate preparation and immunoprecipitation were performed according to the procedures described by Satoh et al. [10]. After incubation, the immune complexes were collected, washed, and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the procedure of Laemmli [12]. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB). For Western blot and lectin blot analyses, after electrophoresis, the separated proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were first blocked with 1% bovine serum albumin (BSA) and then incubated with a biotinylated lectin at a final concentration of 10 μg/m at room temperature for 1 h. Each blot was washed three times and subsequently incubated with horseradish peroxidase (HRP)-conjugated biotin and avidin complex (Vector Labs, Burlingame, CA) at a final dilution of 1:100 at room temperature for 1 h. The membrane was washed three times and then developed with 0.5 mg/ml 4-chloro-1-naphthol containing 0.01% hydrogen peroxide.

2.5. Flow cytometric analysis

All the procedures were performed at 4°C. For cytoplasmic staining on flow cytometric analysis, cells were fixed with 1% formalin and then permeabilized with 0.1% Triton X-100. The cells were washed with PBS, incubated with monoclonal antibody AS17 at a final concentration of 1 μg/ml with 0.5% BSA for 30 min, washed with PBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti mouse IgG (Cappel Labs., Durham, NC) at a final dilution for 1:200 for 30 min. For analysis of cell surface sugar chains, untreated cells were incubated with biotinylated lectins (Seikagaku Co., Tokyo, Japan) with 0.5% BSA, for 30 min at a final concentration of 10 μg/ml, washed with PBS containing 0.5% BSA and then incubated with avidin DCS-FITC (Vector) at a final dilution for 1:200 for 20 min. The cells were washed with PBS containing 0.5% BSA and then resuspended in 100 μl of PBS. Flow cytometric analysis was performed using a flow cytometer, model Cyto ACE (Jasco, Tokyo, Japan). The excitation wavelength was 488 nm with an argon-ion laser, and green fluorescence from FITC collected through a 530 nm band-pass filter was measured on a log scale. Controls consisted of cells treated with normal mouse IgG or without biotinylated lectins.

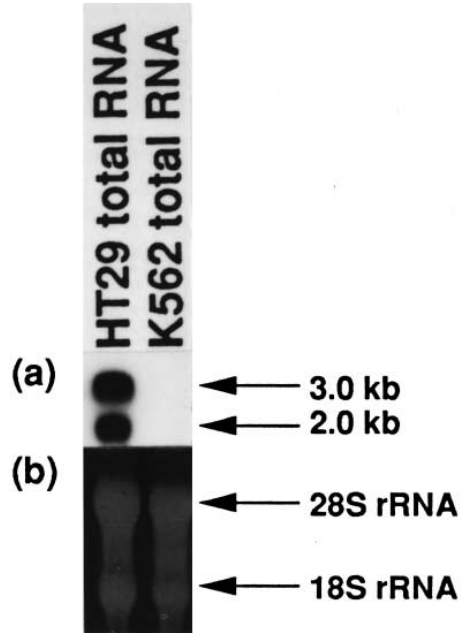

Fig. 1. Northern analysis of annexin IV mRNA. 10 μg of total RNA from human colon adenocarcinoma cell line HT29 and human erythroleukemia cell line K562, respectively, was hybridized with the human annexin IV 2.0 kb cDNA. RNA species of 2.0 kb and 3.0 kb were detected for HT29 but not for K562 cells (a). The RNA was stained with ethidium bromide in the gel prior to blotting (b).

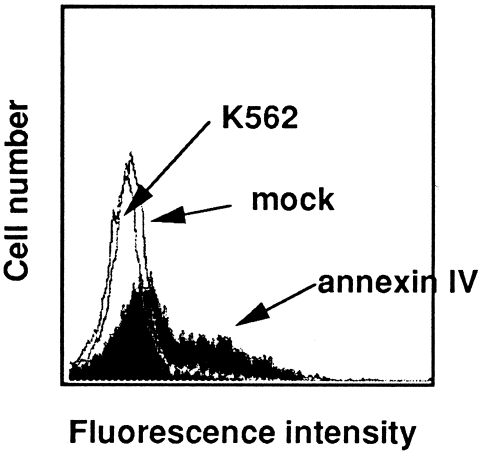

Fig. 2. Expression of annexin IV in transfected K562 cells. Cells expressing annexin IV were subjected to flow cytometric analysis. The cytoplasms of annexin IV cDNA transfectants (annexin IV), vector only transfectants (mock), and normal K562 cells (K562) were stained with monoclonal antibody AS17.

2.6. Reverse transcriptase-mediated polymerase chain reaction analysis of α-2,3-sialyltransferases

Total RNA was extracted from 5×10^6 cells. First-strand cDNA was synthesized with an oligodeoxythymidylic acid primer and avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) from 1 μg of total RNA in a total volume of 100 μl. Second-strand synthesis and amplification were carried out on 1 μl of the first-strand reaction mixtures for sialyltransferase (ST3 and ST4, [13,14]) amplifications and 0.1 μl for β-actin amplification. The reaction mixtures contained 25 pmol of primers and 5 units of Taq DNA polymerase. Thirty amplifications were performed. Each cycle consisted of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C. Aliquots of each reaction mixture were separated by 1% agarose gel electrophoresis. Control PCR reactions were also performed on the first-strand cDNA which had been prepared without reverse transcriptase to exclude the possibility of amplification of contaminating genomic DNA. No amplification of contaminating genomic DNA was detected. The sizes of the fragments amplified from ST3 and ST4 were 481 base pairs (50–530) and 402 base pairs (579–582), respectively, with synthetic oligonucleotide primer.

3. Results and discussion

The size of the mRNA encoding annexin IV was determined by Northern blot analysis. Total RNAs from human colon cancer cell line HT29 and human erythroleukemia cell line K562 were separated in an agarose gel containing formaldehyde, blotted onto a Gene Screen Plus membrane (DuPont NEM Products, Boston, MA), and subsequently hybridized with a probe prepared with human annexin IV 2.0 kb cDNA. The 2.0 kb annexin IV cDNA sequence data are available in the EMBL/GenBank DDBJ under accession number D78152. Total RNA of HT29 gave strongly stained bands at about 2.0 kb and 3.0 kb, but RNA from K562 showed no staining (Fig. 1).

K562 cells were transfected with annexin IV cDNA, since K562 cells do not express either annexin IV mRNAs (Fig. 1) or protein. The annexin IV cDNA transfectants were found to

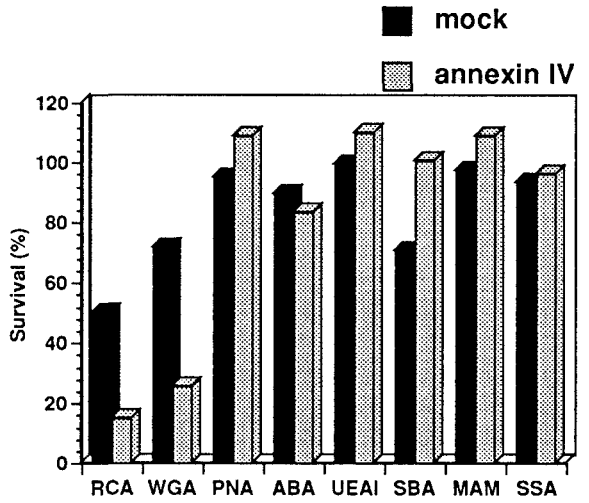

Fig. 3. Lectin toxicity in K562 cell transfectants. Annexin IV cDNA transfectants (annexin IV) and mock transfectants (mock) were treated with biotinylated lectins, and then the cells were analyzed by Cyto ACE. The viable cell numbers are presented as percentages of total cells. The values are means of the results of three experiments.

express annexin IV on flow cytometric analysis with monoclonal antibody AS17 (Fig. 2). The transfectants were cloned by limited dilution, and annexin IV expression was confirmed by immunoprecipitation using AS17.

The annexin IV cDNA transfectants of K562 were found to be slightly more adhesive to culture dishes compared to the wild type and mock transfectants (data not shown), suggesting that cell surface molecules changed on annexin IV cDNA transfection. Previously, we reported that annexin IV exhibits calcium-dependent carbohydrate binding activity [10,15–17]. Furthermore, not only annexin IV but also annexins V and VI were found to have carbohydrate binding properties with different specificities [18,19]. Therefore, we examined whether or not cell surface sugar chains changed on annexin IV cDNA transfection. The transfectants and mock transfectants were treated with biotinylated lectins and then subjected to flow cytometric analysis. The cells expressing annexin IV were more sensitive to *Ricinus communis* agglutinin (RCA120) and wheat germ agglutinin (WGA) than mock transfectants (Fig. 3). The sensitivities of annexin IV transfectants to RCA120 and WGA were 3.3 and 2.8 times greater than those of mock transfectants, respectively. RCA120 and WGA are well-known as cytotoxic lectins that induce tumor cell apoptosis [20]. The increased sensitivities may have been caused by an increase of these lectin receptors on the cell surface. However, since the numbers of lectin receptors were similar in annexin IV and mock transfectants (Fig. 4), the increased sensitivities must be caused by activation of the endocytic pathway, described below. Some annexins have been reported to be involved in the endocytic pathway. Annexin VI is required for coated-vesicle budding, whereas annexin II plays a role in endosomal fusion [21]. It seemed possible that annexin IV may also be involved in the endocytic pathway as annexin II and annexin VI are. The endocytic pathway including ligand-receptor complex uptake and/or endosomal vesicle fusion may be activated by annexin IV transfection.

Furthermore, the transfectants expressed more sugar chains which bind to *Ulex europaeus* agglutinin I (UEA-I) and *Maackia amurensis* mitogen (MAM), which recognize α-1,2-fucosylated and α-2,3-sialylated chains, than mock transfectants (Fig. 4). The expression of UEA-I and MAM by annexin IV transfectants was 1.9 and 2.1 times greater than that by mock transfectants, respectively. MAM binds to sialyl-α-2,3-linked galactosides on tri- and tetra-antennary oligosaccharides [22]. Annexin IV binds not only to glycosaminoglycans but also to sialoglycoproteins such as calf fetuin [15], which has three *N*-linked sugar chains containing highly sialylated tri-antennary oligosaccharides [23]. To identify the proteins that act as endogenous annexin IV ligands, we performed lectin blot analysis of the transfectants and found that a sia-

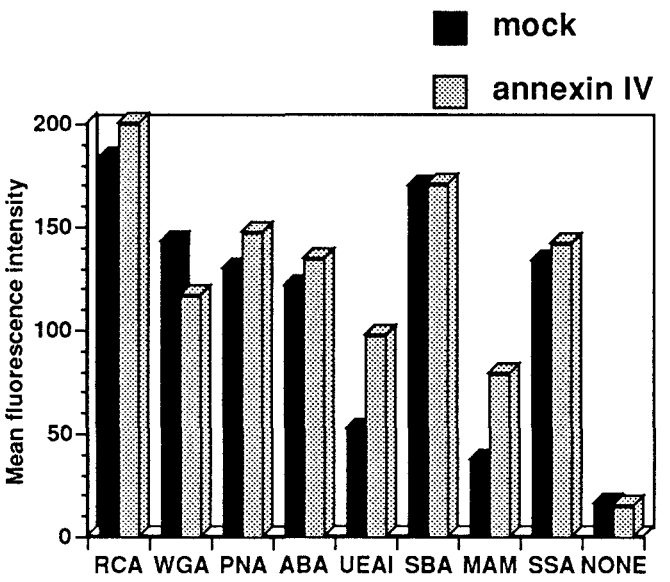

Fig. 4. Differences in cell surface carbohydrate between annexin IV cDNA transfectants and mock transfectants. Annexin IV cDNA transfectants (annexin IV) and mock transfectants (mock) were treated with biotinylated lectins, and then the cells were analyzed by Cyto ACE. The data presented are the mean fluorescence intensities on a logarithmic scale of the viable populations of transfected cells. The values are means of the results of three experiments.

lylated (MAM reactive) glycoprotein of 65 kDa in size was expressed at a slightly higher level by the transfectants than in mock transfectants (data not shown). However, this difference alone cannot explain the results of flow cytometric analysis with MAM. The increase in sialylated and fucosylated sugar chain expression observed following annexin IV transfection may occur for the following reasons: (i) the sialylated and/or fucosylated glycoproteins may be protected from proteolytic degradation on binding with annexin IV; (ii) the cell surface expression of the sialylated and/or fucosylated sugar chains may be increased by membrane-related events such as exocytosis and vesicle transport, in which annexin IV is involved; and (iii) the surface sugar chains may be more sialylated and/or fucosylated due to increased sialyltransferase/fucosyltransferase expression and activity. Some sialyl-α-2,3-linked galactoside sialyltransferases have been cloned and characterized [13,14,24,25]. We therefore examined whether or not sialyl-α-2,3-linked galactoside sialyltransferase expression is increased by annexin IV transfection using RT-PCR methods, but no significant difference was found between the annexin transfectants and mock transfectant and normal K562 cells (data not shown).

Further work is required to determine how annexin IV transfection increases the cell surface expression of sialylated and/or fucosylated sugar chains and, furthermore, whether or not direct interaction between annexin IV and the sialylated glycoproteins is involved in the increased surface expression.

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