Analysis of Extracellular Matrix Proteins in Malignant Chicken Cell Lines

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ABSTRACT. The adherent chicken cell line, MDCC-MSB1-41C, was highly transplantable and metastatic *in vivo*, compared with the parental non-adherent cell line MDCC-MSB1 from Marek's disease (MD) lymphoblastoid tumor. For clarification of differences in extracellular matrix proteins in MSB1-41C and MSB1 cells, examination was made of various components of extracellular matrix proteins. A detachment experiment indicated the protein(s) recognizing the Arg-Gly-Asp (RGD) sequence, the minimum structure required for recognition by the cell-surface receptors, is essential for the adherent character. Immunoblot assay using antibodies showed increased expression of fibronectin, fibronectin receptors, and vinculin on MSB1-41C cell lines. RGD-directed integrins mediate important cell-cell adhesive interaction and these interactions with extracellular matrix proteins may thus possibly be requisite for migration, proliferation and metastatic dissemination of MDCC-MSB1-41C cells. The RGD-containing peptide in the culture medium could cause detachment of cultured adhesive lymphoid leukosis LSCC-1104X5 cells from the dish too. — **KEY WORDS:** chicken cell line, fibronectin, vinculin.

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A Marek's disease (MD) lymphoblastoid T cell line, MDCC-MSB1-41C (MSB1-41C), was highly transplantable and metastatic *in vivo*, compared with the parental cell line MDCC-MSB1 (MSB1) [16]. Important differences between these two tumor cell lines are the capacity to develop tumors and to adhere to the culture dish [16]. In this report, we have contrasted the biochemical properties of adherent MSB1-41C and non adherent MSB1 cell lines. Two independent lymphoblastoid lines of B cell origin (LSCC-RP9 and LSCC-1104X5) and chicken embryo fibroblast (CEF) cells were compared with these Marek's disease derived cell lines.

Four chicken lymphoblastoid cell line, adherent MSB1-41C [16, 21] and 1104 X5 [12] lines and non-adherent MSB1 [1] and RP9 [17] lines, were cultured in RPMI 1640 medium (RPMI), supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). CEF cells free from endogenous virus were prepared from fertile eggs obtained from Nippon Institute of Biological Science, Yamanashi, Japan. They were cultured in Eagle's medium (MEM), supplemented with 5% heat-inactivated fetal bovine serum, 10% tryptose phosphate broth (TPB), glutamine, penicillin and streptomycin as described previously [15].

To assess the specificity of the adhesion of adherent cells (MSB1-41C, 1104X5, and CEFs), the cells were incubated on tissue culture dishes (Falcon) in the presence of a peptide having the sequence Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP). The RGD sequence can be used to detach cultured cells from the dish, because many of cell adhesion receptors, such as the integrins, recognize simple sequences that can be produced as synthetic peptides and a number of integrins recognize the RGD sequence [11, 19, 20].

The detaching effects of soluble peptides on cultured cells were studied. When the hexapeptide GRGDSP (RGDpeptide) was added to routine cultures of CEFs, MSB1-41C, and 1104X5 cells at 1 mg/ml (1.5 mM), all

three cell types became detached from the surface of the dishes after 1 hr (Fig. 1A, C, and E). This appeared to be specific since the control peptide GRGESP containing an Asp-to-Glu substitution had no evident effect on the cells at the same concentration (Fig. 1B, D, and F). In the case of CEFs, holes subsequently appeared in the monolayer as the cells retraced into ridges. However, even after a few days, the cells still had not become detached from the culture

Fig. 1. Effects of synthetic peptide on cell cultures. Routine cultures of normal chicken embryo fibroblasts (A, B), MDCC-MSB1-41C (C, D), and LSCC-1104X5 (E, F) cells were treated by adding 1 mg/ml of the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro (A, C, E) or Gly-Arg-Gly-Glu-Ser-Pro (B, D, F) by addition to the culture medium for 1 hr. Cells were plated 24 hr before this addition at a density that gave 70% confluent culture when the peptide was added.

plate completely, similar to Fig. 1B. MSB1-41C and 1104X5 cells detached from the plate completely. For MSB1-41C, the detached cells didn't float separately in the medium but formed aggregations (Fig. 1C). 1104X5 cells floated in the medium individually (Fig. 1E).

This result strongly suggests that these cells bind specifically by special molecules, the so-called RGD-directed integrins. It is not clear at this time which molecules mediate cell-dish interactions hindered by the RGD peptide. The RGD sequence was identified first in fibronectin. It is known in the insoluble form that fibronectin can interact with cells effecting cell attachment. In order to examine the distribution of fibronectin, immunofluorescence staining of adherent cells was made by the fibronectin antibody (Chemicon AB1946) prepared against chicken fibronectin [2]. Cells were plated in 4-well Lab-Tek chambers (Miles Sci., Naverille, IL) fixed for 4 min in -20°C MeOH, quickly rinsed in -20°C acetone and allowed to air dry as described previously [9]. Serial dilution of anti-fibronectin serum showed positive staining at maximal 1/100 dilution. All chambers were treated with Protein Blocking Agent (BBA-Universe, Lip Shaw) and then anti-fibronectin serum 1/100 in PBS and incubated for 40 min at 37°C in a humid chamber. After washing in PBS, a drop of anti-rabbit Ig antiserum conjugated with FITC in PBS was placed in each chamber. After 40 min at 37°C , the specimens were thoroughly rinsed and mounted in 50% glycerol PBS and examined on a Zeiss Axiophot microscope. Controls on which preimmune rabbit serum was used were consistently negative. In agreement with earlier studies on chicken fibroblast [2], fibronectin was readily detected at the cell surface and extracellular matrix of CEF by immunofluorescent staining. Adherent MSB1-41C and 1104X5 cells showed weaker staining and confirmed the presence of fibronectin. Fibronectin-fibrils surrounding cell lines were more slender, and overall staining was less intensive than that noted for CEFs (Fig. 2). Clusters of isolated thicker fibrils were present in MSB1-41C (Fig 2B) infrequently. 1104X5 cells contained even fewer fibrils and a very weak fibrillar fibronectin network (Fig. 2C).

Possible quantitative alteration is considered in some extracellular matrix proteins to be an explanation for their attachment to the dishes, so fibronectin, fibronectin receptors and vinculin were determined for these cell lines by immunoblot assay using antibodies reacting with proteins. The cell cultures were washed with ice-cold Tris-buffered saline, scraped off the plate, quickly centrifuged and frozen with dry ice and ethanol. The frozen pellets were suspended in Sol buffer (10 mM Tris hydrochloride, pH7.4, 1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 0.1 mM sodium molybdate and 1 mM phenylmethly-sulfonyl fluoride) and then immediately heated in boiling water for 5 min. The protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories). Forty microgram of proteins were analyzed by the SDS-10% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred electrophoretically to a Trans blot (Bio-

Fig. 2. Indirect immunofluorescent staining of adherent cell lines with anti-fibronectin antiserum: (A) normal chicken embryo fibroblasts: (B) MDCC-MSB1-41C cells: (C) LSCC-1104X5 cells. Figure 2B and 2C cells were photographed by exposure of 10s ($\times 200$). Figure 2A cells were photographed by exposure of 2s owing to the greater fluorescence ($\times 200$).

Rad Laboratory) filter by a Semi dry electroblotter (Sartorius), and stained with each antibody and then alkaline phosphatase-conjugated anti-IgG antibodies and BCIP/NBT phosphatase substrate kit (Kirkegaard & Perry Lab. Inc.) as described before [5, 13]. Fibronectin detected as a 220 k dalton band by antiserum AB1946 increased somewhat more in adhesive cell lines (MSB1-41C and 1104X5) than in suspended lymphoblast cells (MSB1 and RP9), though the expression was much less than in normal CEFs (Fig. 3A). Fibronectin-receptors detected as a 140 k dalton-broad band by the antiserum AB1067 (Chemion) increased in adhesive MSB1-41C cells compared to parental MSB1 cells, though the expression of fibronectin-receptors in 1104X5 cells and RP9 cells was essentially low (Fig. 3B). The expression of vinculin detected as a 130 k dalton band by the monoclonal VIN-11-5 (BioMaker) [8] increased much more in MSB1-41C than in the parental MSB1 cells (Fig. 3C), but was basically the same for RP9 and 1104X5 cells.

To obtain evidence of altered gene expression which is responsible for the acquisition of metastatic competency, we examined the biochemical differences of cell surface proteins between the low (MSB1) and high metastatic chicken lymphoma cells (MSB1-41C). We found that the RGD peptide could cause detachment of high metastatic MSB1-41C cells. This result suggests that MSB1-41C cells express more functional RGD-dependent cell adhesion receptors than the parental MSB1 cells. We also found that the expression of vinculin increased significantly in the MSB1-41C cell line, and a slight increase in the amount of fibronectin and fibronectin-receptor was also observed in the high metastatic MSB1-41C, compared with parent MSB1 cells. In addition, the RGD peptide could cause detachment of other chicken adherent cultured cells: B cell originated 1104X5 cells and CEFs.

Though we currently have no data concerning the influence of cell surface proteins on metastatic activity, our report offers a new probe for a better understanding of the metastatic activity of chicken lymphoma cell lines. The effect of the RGD peptide on cells plated on culture dishes suggests that the RGD-directed integrins participate in their

Fig. 3. Immunoblot analysis of cell extracts of normal chicken embryo fibroblasts, and chicken lymphoblastoid cell lines with anti-fibronectin serum, anti-fibronectin receptor serum and anti-vinculin monoclonal antibodies. Equal amounts (40 μ g) of total protein lysate were separated by electrophoresis and blotted onto Trans Blot (PVDF) filters. Fibronectin (A), fibronectin-receptor (B), and vinculin (C) were determined by quantitative immunoblotting as described in the text. Their molecular weights were determined from the positions of protein molecular mass standards (Pharmacia P-L Biochemicals Inc.). (1) Normal chicken embryo fibroblasts. (2) MDCC-MSB1 cells. (3) MDCC-MSB1-41C cells. (4) LSCC-RP9 cells. (5) LSCC-1104X5 cells.

adhesion to the dishes. Since the detaching effect of the RGD peptide is specific and not due to toxicity, detached cells reattached and grew after removal of the peptide (data not shown). It has been suggested that treatment of cells with RGD peptide can cause some integrins to relocate [3]. We think the RGD peptide is useful *in vitro* as a research tool. To examine if intravenous co-injection of RGD peptide with MSB1-41C cells can reduce metastasis is under way. The RGD sequence is the essential element of the integrin recognition site in many different adhesion proteins [3]. The adhesion proteins with active RGD sequences include fibronectin, vitronectin, laminin, etc. Fibronectin contains 220 k-dalton subunits linked into dimers. It binds to the cell surface as well as other extracellular molecules. We observed a moderate increase of fibronectin in MSB1-41C cells, and this may possibly be connected with cell adhesion. Since vitronectin is abundant in serum and important in mediating the attachment of cultured cells [3], vitronectin and other proteins as adhesive components may be needed to further dissect the possible interactions among these proteins. Very little has been reported about chicken integrins, although Horwitz *et al.* showed the existence of a chicken fibronectin receptor by a monoclonal antibody [14]. This monoclonal antibody could perturb cell substrate attachment, and the receptor bound to fibronectin and laminin and vitronectin. In MSB1-41C cells, the RGD peptide can cause detachment, and the expression of the fibronectin-receptor increased more than in its parent cell line. The fibronectin-receptor could be the target of the RGD peptide, and this requires further study. It is known

that circulating murine lymphocytes lack the ability to attach fibronectin, but lymphocytes isolated from the murine thymus do have receptors and do attach to fibronectin [4, 18]. There is a possibility that the MS1-41C line is derived from an immortalized cell of a certain leukocytic lineage. If this is so, then MSB1 and MSB1-41C might be useful for study of chicken T cell differentiation. The fibronectin receptor may anchor both fibers to opposite sides of the plasma membrane. The fibronectin receptor becomes immobilized at sites in contact with the substrate and keeps cells tightly bound to the matrix. The immobilization may be due to binding of the receptore, via vinculin or some other protein, to the actin cytoskeleton [3]. In high metastatic MSB1-41C, the expression of vinculin was shown to increase markedly. Vinculin is an ubiquitous 130 k-dalton protein associated with patches called adhesion plaques on the cell membrane and cell-cell contact, at the termini of microfilament bundles [7]. Fernandez *et al.* [6, 7] suggested high expression of vinculin affected cell morphology, metastases dramatically and suppressed the ability of the cells to form tumors after transfection when injected into mice cells. In MSB1-41C cells the increase in vinculin expression may contribute to substrate adhesiveness. Still the cause-and-effect relationship between vinculin regulation, cell attachment and ability of cells to form tumors remains to be determined.

Better characterization of specific intercellular adhesive molecules and cell-to-cell interaction in MDCC-MSB1-41C cells will lead to greater clarification of the invasiveness and spread of malignant tumor cells.

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