Interaction between 120 kDa Fragment in β-Subunit from Egg White Ovomucin and Sarcoma-180 Cells through Basic Fibroblast Growth Factor Receptor

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The mechanism of antitumor activities in hen egg white ovomucin (OVM) was analyzed by examining the interaction between the 120 kDa fragment (a highly glycosylated fragment in β-subunit from the pronase-treated OVM) and basic fibroblast growth factor receptor (bFGFR). The 120 kDa fragment, which was transblotted onto a polyvinylidene difluoride membrane after SDS-polyacrylamide gel electrophoresis, was found to interact with bFGFR. The interaction was detected by Western blotting method with mouse anti-FGFR IgM. When sarcoma-180 (SR-180) cells were cultured on addition of the 120 kDa fragment, they were observed to be in the necrotic state by transmission electron microscopy, and the 120 kDa fragment was seen to be dispersed on the cell surface by light and transmission electron microscopic observations with the immunocytochemical staining method. The growth of SR-180 cell in the presence of bFGF was reduced with increase in the amount of 120 kDa fragment added. Thus, it was found that the 120 kDa fragment interacted with SR-180 cells through bFGF and affected them.

Keywords: egg white protein, ovomucin, SR-180 cells, basic fibroblast growth factor receptor

Hen egg white ovomucin (OVM), which accounts for about 3.5% of the protein in egg white, is a heavily-glycosylated glycoprotein which consists of α- and β-subunits with a macromolecular structure bound by disulfide bonds between their subunits. In our previous reports on antitumor activities of OVM, β-subunit from OVM was shown to have a cytotoxic effect on cultured tumor cells such as SEKI cells (human melanoma cell) and 3LL (Lewis lung cancer cell) by scanning electron microscopy (Ohami et al., 1993). Moreover, the antitumor effects of fragments (220 and 120 kDa-highly glycosylated fragments in β-subunit) separated from pronase-treated OVM were demonstrated in a “double grafted tumor system”, using Meth-A fibrosarcoma cells (Watanabe et al., 1998a). To further clarify the cytotoxic activity of OVM in the in vitro system, we described the growth inhibition and morphological cell damage of sarcoma-180 (SR-180) cells cultured with β-subunit rather than α-subunit prepared from OVM (Yokota et al., 1999a). Then, in a subsequent report on xenografted SR-180 cells in mice, the light and transmission electron microscopic features of tumor portions showed that β-subunit-treated tumor cells were in a degenerated and necrotic state (Yokota et al., 1999b).

These results indicated that subunits and fragments from OVM exert direct cytotoxicity on the tumor cells, and an indirect cytotoxic effect via the host’s immune system. The direct growth inhibition suggested that samples might adhere specifically to tumor cells as described in previous papers (Ohami et al., 1993; Yokota et al., 1999a). Thus, the OVM subunits and fragments proved to be effective against some tumors, yet the mechanism of action is not yet clearly understood.

Fibroblast growth factors (FGFs, polypeptide growth factors) are potent growth and angiogenic factors which are abundant in normal and malignantly transformed cells. Basic FGF (bFGF), a representative growth factor among FGFs, is well correlated with cell proliferation, tumor progression and angiogenesis (Folkman & Klagsbrun, 1987; Rifkin & Moscatelli, 1989). It was speculated to be one of the mechanisms by which the OVM subunits and fragments exerted antitumor activity by inhibiting the growth-promoting action of FGFs, that is, the inhibition of the binding of FGF to the tumor cells by interaction of the FGF receptor (FGFR) with OVM subunits or fragments.

We are currently studying the effects of administration of OVM and its fragments on antitumor activities for their possible use as food-physiologically active substance. This paper provides evidence on the interactions between the 120 kDa fragment and bFGFR using the Western blotting detection method, and between this fragment and the SR-180 cells through bFGF using light and transmission electron microscopes.

Materials and Methods
Preparation of pronase-treated fragments and separation of 120 kDa fragment OVM from fresh egg white (White Leghorn hens) was prepared as a gel-like precipitate by the method of Kato et al. (1970) and freeze-dried. OVM dispersed in K-phosphate buffer (pH 8.5) was incubated with pronase (from Streptomyces griseus, Calbiochem-Novabiochem Co.) for 24 h at 37°C at a ratio of 1/6400 (pronase/OVM, w/w), inactivated by heating at 100°C for 5 min and centrifuged at 6000 rpm for 30 min to obtain the supernatant, which was then dialyzed against water, freeze-dried and used as a pronase-treated fragment. Next, the fragment was applied to gel filtration (Sephacryl S-400, 2.0× 73.0 cm, Pharmacia LKB, Uppsala, Sweden). The separated 120...
kDa fragment-containing fraction was dialyzed against water and freeze-dried. This material was again treated using the same procedure to obtain the purified 120 kDa fragment. These procedures were carried out according to the method described previouantly (Tsuge et al., 1997b; Watanabe et al., 1998b).

**Materials**

SR-180 cells were obtained from the American Type Culture Collection (USA). The human FGFR extracellular domain (recombinant) which is able to bind bFGF and inhibit bFGF-mediated proliferation of cells was obtained from Austral Biologicals (California, USA) and used as bFGFR. Recombinant bovine bFGF, which is effective on mouse, rat, rabbit, bovine and human cells, was obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). The mouse anti-120 kDa fragment antibodies were prepared as described (Tsuge et al., 1997a), and treated with protein A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). The purified antibodies were used as the mouse anti-120 kDa fragment IgG. The mouse anti-FGFR IgM was obtained from CHEMICON International Inc. (California, USA).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed using 7.5% gel according to the method of Laemmli (1970). Portions of 20 μl of the samples (0.1%), which were prepared in Tris-glycine buffer with 2-mercaptoethanol (2-ME) and heated at 100˚C for 3 min, were applied to each slot of gel and electrophoresed at a constant current of 17 mA. The gels were stained with Coomassie Brilliant Blue R-250 (CBB) for the detection of protein and with periodate-Schiff (PAS) reagent for the detection of carbohydrate.

**Western blotting analysis**

The 120 kDa fragment was electrophoresed on SDS-polyacrylamide gel, then electropherated onto PVDF (polyvinylidene difluoride) membrane (Bio-Rad Laboratories, Hercules, CA) by the semi dry electrophorating method (Matsudaira, 1987). The membrane was incubated with 10 ml of FGFR solution (1 μg/ml). The interaction between 120 kDa fragment and FGFR was detected with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Tokyo) according to the manufacturer’s protocol, after the reactions with mouse anti-FGFR IgM, followed by incubation with a Histofine SAB-PO(M) kit (Nichirei, Tokyo).

**Cell proliferation**

Cultured SR-180 cells were suspended in 3.2 ml of RPMI 1640 medium with 10% FCS at a density of 1×10⁶ cells/ml, and 160 μl of 120 kDa fragment (10 mg/ml) was added to the dishes, followed by incubation at 37˚C for 6 h. The cells were carefully harvested and thoroughly washed with phosphate-buffered saline (PBS).

**Transmission electron microscopy**

The washed cells were prefixed with 2.5% glutaraldehyde in PBS for 60 min followed by three washings with PBS, and then postfixed with 1% osmium tetroxide for 30 min. The fixed cells were dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections treated with lead citrate staining were ages of ethanol up to absolute ethanol and embedded in epoxy resin. Ultrathin sections were applied to each slot of gel and electrophoresed at a constant current of 17 mA. The gels were stained with Coomassie Brilliant Blue R-250 (CBB) for the detection of protein and with periodate-Schiff (PAS) reagent for the detection of carbohydrate.

**Inhibition of cell growth by 120 kDa fragment**

SR-180 cells were seeded into 96-well culture plates at a density of 1×10⁶ cells per well, and incubated with fetal bovine serum-free RPMI 1640 medium at 37˚C for 48 h with and without various amounts of 120 kDa fragment (0, 25, 50, 100 μg/well) and the addition of bFGF (10 ng/well). As a control, SR-180 cells were similarly incubated with the medium described above without the additions of 120 kDa fragment and bFGF. The cell counts were measured by MTT assay (Mosmann, 1983).

**Results**

The characteristics of the chemical composition of 120 kDa fragment used in this study were shown in a previous paper (Tsuge et al., 1997a; Watanabe et al., 1998a). Briefly, the fragment was a carbohydrate-rich protein with O-linked oligosaccharides (protein content=about 10%, carbohydrate content=about 90% containing N-acetylgalactosaminic acid, NeuAc of 28%).

The 120 kDa fragment, which was electrophoresed on SDS-polyacrylamide gel and transblotted onto PVDF membrane, was reacted with FGFR. As seen in Fig. 1, an interaction between the fragment and bFGFR was observed, although the mode of this interaction was unknown.

The morphological changes of SR-180 cells cultured with and without 120 kDa fragment were seen in transmission electron microscopy.
microscopic observations (Fig. 2). It was noted that almost all the SR-180 cells treated with 120 kDa fragment showed the changes of irregular clumping of chromatin, irregular nuclear shape and marked swelling of organelles in cytoplasm, most likely due to the development of necrosis. Membranes were subsequently disintegrated, but the cells usually retained their overall shape (Fig. 2-A). On the other hand, SR-180 cells (control) without 120 kDa fragment (Fig. 2-B) contained very dense intracellular materials with intact cytoplasmic membranes, indicative of healthy cells. These changes appeared similar to those previously described (Yokota et al., 1999a), in which SR-180 cells were treated with β-subunit consisting of 120 kDa and 220 kDa fragments. Thus, these observations suggested that 120 kDa fragment was a main constituent in β-subunit for the development of morphological changes, and such changes might derive from the interaction between the cell membrane and this fragment.

It is generally known that there are some FGFRs on the surface of the tumor cells which acquire growth factors. In this study whether or not 120 kDa fragment and the anti-FGFR IgM interact with bFGFR on the SR-180 cell surface were examined by the immunocytochemical staining method, respectively.

Figure 3 shows the light microscopic observations of the immunocytochemically stained SR-180 cells. The stained appearance of these cells revealed that 120 kDa fragment interacted with almost all of them (Fig. 3-A), while control without the 120 kDa fragment was not visualized (data not shown). On the other hand, an interaction between the cells and mouse anti-FGFR IgM was also detected, as seen in Fig. 3-B, indicating that there are some FGFRs on the cell surface which were able to react with mouse anti-FGFR IgM.

Figure 4 shows the transmission electron microscopic features after the immunocytochemical staining of the SR-180 cells cultured with 120 kDa fragment, which were treated in a manner similar to the cells in Fig. 3. Localization by immunocytochemical staining of 120 kDa fragment revealed electron-dense reaction products at the cell membrane surface (Fig. 4-B). The SR-180 cells treated in the same manner without the 120 kDa fragment did not show such products (Fig. 4-A). Such differences showed that 120 kDa fragment interacted with FGFR on the surface of SR-180 cells, although whether or not such interactions were specific was not clarified by these results. Figure 4-C also shows the immunocytochemical staining features, which did not reveal electron-dense reaction products after the treatment with anti-FGFR IgM of the SR-180 cells cultured with the 120 kDa fragment. This result, together with Fig. 3-B showing the interaction of FGFR on the SR-180 cells with anti-FBFR IgM, indicated that the binding of anti-FGFR IgM to the FGFR on SR-180 cells was blocked by the presence of the 120 kDa fragment, i.e., the fragment interacted with FGFR on the cell surface.

From the observations described above, the inhibition of growth of SR-180 cells was presumed from the interactions between bFGFR on the cell surface and the 120 kDa fragment. The changes in SR-180 cell proliferation based on the treatment...
with the 120 kDa fragment in the presence of bFGF were examined by MTT assay, as shown in Fig. 5. The results of this experiment indicate that cell growth in the presence of bFGF was inhibited by the 120 kDa fragment in a dose-dependent manner. It might be concluded that the binding between bFGFR and bFGF, which is required for the cell growth, was partly inhibited by the interactions between bFGFR and the 120 kDa fragment.

Discussion

It was presumed from the results of antitumor activities in our previous papers as described in the Introduction that the bindings of OVM subunits (or their fragments) to the tumor cells were important for cytotoxic effects. In this paper the 120 kDa fragment which was derived from β-subunit was shown to interact with bFGFR (Fig. 1) and also to interact with bFGFR on the SR-180 cell surface (Figs. 3 and 4), suggesting an inhibiting effect on the supply of FGF to the SR-180 cells based on the suppression of binding between bFGF and bFGFR on the cell surface, that is, the inhibition of cell proliferation (Fig. 5). On the other hand, the SR-180 cells incubated with 120 kDa fragment resulted in necrosis (Fig. 2). It is natural to assume as a factor of necrosis that the supply of oxygen and nutrients to the SR-180 cells and the excretion of waste from them were inhibited by the binding of 120 kDa fragment.

A putative autocrine/paracrine role for the FGF/FGFR system has been suggested in the mitogenic activation of fibroblasts or myofibroblasts (Wilson et al., 1993; Rosenbaum et al., 1995). It has also been reported that the autocrine growth stimulation between bFGF and FGFR appears to increase the severity of biological malignancy of tumor (Hasebe et al., 1997). The inhibition of cell growth in the present investigation showed that the autocrine/paracrine role might be blocked by the addition of the 120 kDa fragment.

Rallet et al. (1987) showed that OK-432 (Picibanil), an inactivated preparation from Streptococcus pyogenes which is now used as a cancer chemotherapeutic agent, bound specifically to the tumor cell surface membrane and was phagocytized by tumor cells, thus bringing about a growth inhibitory effect on tumor cells. In the present study, the binding between 120 kDa fragment and SR-180 cells also appears essential to the expression of the cytotoxic activities. However, it remains unknown what post-binding mechanisms are involved in 120 kDa fragment-mediated cytotoxicity. The transmission electron microscopic observations (Fig. 4) did not show that the 120 kDa fragment interacting with bFGFR on the SR-180 cell surface was phagocytized by the cells. This suggested that the action of this fragment was exerted from the surface of SR-180 cells. The 120 kDa fragment bound directly to the surface of the cells caused a disorder in the intracellular microapparatus and cellular metabolism, resulting in inhibition of DNA synthesis.
bFGF (polypeptide of 146 amino acid residues) binds to heparan sulfate proteoglycan at the cell surface and to a receptor with tyrosine kinase activity (Rapraeger et al., 1991). The 120 kDa fragment is O-glycoprotein containing clustered sialic acid (NeuAc). It may be natural to think that the carbohydrate chain in glycopeptides such as 120 kDa fragment in addition to β-subunit in OVM is related to the interaction with FGFR. The concept has gradually emerged that the carbohydrate moieties of glycoconjugates might act as recognition signals (Pilatte et al., 1993). However, in the previous study (Watanabe et al., 1998a), it was found using the desialylated 120 kDa fragment that NeuAc residues are not necessarily essential for direct antitumor activity but might be indispensable for indirect activity of this kind. Thus, it could not necessarily be concluded that the carbohydrate chain containing NeuAc residues was directly related to the interaction with the tumor cell surface. Further studies must determine whether the bFGFR binding sites in glycopeptides are either carbohydrate moieties or the peptide portion, or both, together with their molecular structure. Now we are studying the binding site in 120 kDa fragment to SR-180 cells.

There are receptors other than bFGFR, such as acidic FGFR, EGF (epidermal growth factor) receptor and VEGF (vascular endothelial growth factor) receptor, on the tumor cell surface membrane. The 120 kDa fragment might interact specifically or nonspecifically with such receptors in addition to its interaction with bFGFR, thereby leading to necrosis. Therefore, it is also necessary to determine the binding levels of 120 kDa fragment to bFGFR in comparison with the other receptors on tumor cells.

References