Inhibition of cell growth by a fused protein of human ribonuclease 1 and human basic fibroblast growth factor

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Pancreatic-type RNases are considered to have cytotoxic potential due to their ability to degrade RNA molecules when they enter the cytosol. However, most of these RNases show little cytotoxicity because cells have no active uptake mechanism for these RNases and because the ubiquitous cytoplasmic RNase inhibitor is considered to play a protective role against the endocytotic leak of RNases from the outside of cells. To study the cytotoxic potential of RNase toward malignant cells targeting growth factor receptors, the C-terminus of human RNase 1 was fused to the N-terminus of human basic fibroblast growth factor (bFGF). This RNase–FGF fused protein effectively inhibited the growth of mouse melanoma cell line B16/BL6 with high levels of cell surface FGF receptor. This effect appeared to result from prolongation of the overall cell cycle rather than the killing of cells or specific arrest in a particular phase of the cell cycle. Thus, human RNase 1 fused to a ligand of cell surface molecules, such as the FGF receptor, is shown to be an effective candidate for a selective cell targeting agent with low toxic effects on normal cell types.

Keywords: cytotoxic/FGF/immunotoxin/RNase/targeting

Introduction

Improvement of cytotoxic agents specific to cancer cells or other cells causing proliferative diseases is one of the goals of targeting chemotherapy. Such cells often express surface receptors or other molecules distinguishing them from the surrounding normal cells. In this regard, many immunotoxins have been developed which exploit this difference. Immunotoxins are defined as proteins containing an antibody (or a ligand) and a toxin (Lappi and Baird, 1990; Krettmann and Pastan, 1998). The moiety of antibody or ligand specific for a cell surface molecule delivers the toxin to the target cells. Plant and bacterial toxins have been the most intensively studied moieties. These toxins are enzymes that catalytically abolish protein synthesis when they translocate into the cytosol. Although some of these immunotoxins are promising, clinical applications are still limited due to their inherent toxicities to normal cells as well as their immunogenicity. Distinct targeting specificity and humanized design of immunotoxins are needed to overcome these difficulties.

It has been shown that upon microinjection of Xenopus oocytes, bovine RNase A inhibited protein synthesis at concentrations comparable to plant and bacterial toxins (Saxena et al., 1991). Therefore, RNases are considered to be alternative toxins because of their abilities to degrade RNA molecules. In fact, RNase A chemically conjugated to human transferrin and to antibodies to the transferrin receptor (Rybak et al., 1991), or to human epidermal growth factor (EGF; Jinno et al., 1996b) have been shown to be cytotoxic to cells bearing the transferrin receptor or to the EGF receptor (EGF)-overexpressing carcinoma cells, respectively. These results indicate that human enzymes belonging to the RNase A family can be used as toxins in the construction of immunotoxins (Youle et al., 1993), although human RNases as well as RNase A are not in themselves cytotoxic. In humans, six members of the extracellular RNase family are known. These RNases are distributed in various tissues (Rosenberg and Dyer, 1996; Futami et al., 1997) and possess various biological activities in addition to digestive activity (Sorrentino, 1998). We have reported the expression of recombinant human RNase 1 which was cloned from human pancreas (Futami et al., 1995). We have also shown that recombinant RNase 1 chemically conjugated and/or genetically fused to EGF is effective as an immunotoxin against EGF-overexpressing human squamous carcinoma cells (Jinno et al., 1996a; Psarras et al., 1998). Other members of human RNases, such as RNase 2 (eosinophil-derived neurotoxin, EDN) and RNase 5 (angiogenin), have been successfully used as toxic domains of immunotoxins (Newton et al., 1994, 1996; Zewe et al., 1997).

Basic fibroblast growth factor (bFGF) can regulate a number of distinct biological activities in vivo and in vitro (Basilico and Moscatelli, 1992) and is considered to be involved in the growth of a variety of neoplastic cell lines derived from melanomas (Halaban et al., 1988), breast cancer (Penault-Llorca et al., 1995), astrocytoma (Zagzag et al., 1990) and ovarian carcinoma (Crickard et al., 1994), and in the proliferation of mesangial cells in the glomerular disease (Floege et al., 1993). Since overexpression of FGF receptors possibly correlate with the malignancy of tumors and bFGF is proposed to support autocrine growth of melanoma cells (Ahmed et al., 1997), the receptors are potential targets for melanoma therapy (Kato et al., 1992). In fact, the chemical conjugates or fusion protein between bFGF and saporin, a ribosome-inactivating protein isolated from the plant Saponaria officinalis, have been developed to show the growth inhibitory effect on the cells expressing FGF receptors (Lappi et al., 1991, 1994; Beith et al., 1992).

In this paper, we designed a fused protein between human RNase 1 and human bFGF as humanized immunotoxins. The fused proteins showed cell type-specific growth inhibition against malignant cells bearing FGF receptors; however, the effect was cytostatic rather than cytotoxic.

Materials and methods

Materials

Recombinant human RNase 1, des.1-7/RNase 1 and human bFGF [147 amino acid form] were purified from Escherichia coli as described previously (Futami et al., 1995; Iwane
grown in LB medium containing ampicillin (50 µg/ml) overnight at 37°C. The culture was then diluted 50-fold with LB medium and further incubated at 37°C in an incubator shaker. When an $A_{600}$ of 0.6 was reached, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the growth was continued for a further 3 h at 37°C. Cells were harvested by centrifugation and washed once with physiological salt solution, and stored at −80°C until they were used.

Frozen cells obtained from a 1 L culture were thawed at 37°C and suspended in 100 ml lysis buffer (10 mM Tris–HCl, pH 7.5, containing 10% sucrose and 0.2 M NaCl). The suspension was then homogenized (LK-21; Yamato, Japan) followed by freeze–thaw for complete lysis. The insoluble fraction was collected by centrifugation, resuspended in 10 ml lysis buffer, sonicated on ice for 3 min, and then centrifuged at 10 000 g for 10 min. The pellet was washed with 50 ml of 0.5% Triton X-100 containing 1 mM EDTA and 0.2 M NaCl, and then centrifuged at 10 000 g for 10 min. The resulting pellet was dissolved in 10 ml of 0.1 M Tris–HCl, pH 8.5, containing 6 M guanidinium–HCl (GdmCl), and then the fused proteins were reduced with 0.1 M 2-mercaptoethanol at 37°C for 90 min under a nitrogen atmosphere. The solution was diluted to a final protein concentration of 100 µg/ml into redox buffer [10 mM Tris–HCl, pH 8.5, containing 0.5 mM oxidized glutathione (Kojin, Japan), 3 mM reduced glutathione/2-mercaptoethanol, 30% (v/v) glycerol and 0.4 M GdmCl] and then incubated for 12 h at 4°C. After removal of insoluble material by centrifugation, the supernatant was applied to a heparin–Cellulofine (Chisso, Japan) affinity column (150×560 mm) equilibrated with buffer A [30 mM Tris–HCl, pH 7.5, containing 1 mM dithiothreitol (DTT)]. The column was washed with buffer A containing 1.0 M NaCl, and the adsorbed protein was eluted with buffer A containing 2.0 M NaCl. The eluted fractions were pooled and stored at −80°C until use. Usually, the protein solution thus obtained was appropriately diluted and used for biological assay. If necessary, fused proteins were desalted by C$_4$ reverse phase HPLC (Ultron 300 C$_4$, 4.5×150 mm, Chromatopacking Center, Japan) before analysis.

Amino acid compositions of fused proteins were determined by Hitachi L8800 amino acid analyzer after hydrolysis of purified samples, and N-terminal sequences of fused proteins were determined with an Applied Biosystems Procise™ 491 protein sequencer.

**Assay of RNase activity**

RNase activity using poly(C) was measured by a spectrophotometric procedure (Sorrentino and Libonati, 1994) with some modification. Briefly, 495 µl substrate solution [0.1 M HEPES–NaOH, pH 7.5, containing 0.1 M NaCl, 0.01% bovine serum albumin and 0.1 mM (in phosphodiester groups) poly(C)] was pre-incubated at 25°C. The reaction was started by adding 5 µl of the appropriate amount of each RNase or RNase–FGF fused protein and the increase in absorbance at 260 nm due to the transphosphorylation reaction catalyzed by RNase was continuously monitored with a spectrophotometer (Hitachi U-2000, Japan) at 25°C.

**Heparin affinity HPLC**

Purified protein solution was appropriately diluted with buffer A (described above) to reduce the NaCl concentration to 0.2 M, then applied to the heparin affinity HPLC column (Shodex AF pak HR-894, 8×50mm, Showa-denko, Japan). The elution was performed using a linear gradient of NaCl.

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et al., 1987). Single-stranded polynucleotide, poly(C), a preferable substrate for human RNase 1, was purchased from Seikagaku Kogyo (Japan).

**Cell culture**

Mouse metastatic melanoma B16/BL6 cells (Poste et al., 1980) were grown in RPMI 1640 (Nissui, Japan) medium supplemented with 10% fetal bovine serum (FBS). Mouse BALB/c 3T3 A31 cells (Sasada et al., 1988) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Nissui, Japan) supplemented with 10% calf serum (CS). All media contained 70 µg/ml kanamycin as antibiotic and each cell line was maintained in a humidified atmosphere consisting of 5% CO$_2$ in air at 37°C.

**Plasmid construction of RNase–FGF fused proteins**

Human RNase 1 or des.1-7RNase 1 cDNA (Seno et al., 1994; Futami et al., 1995) was ligated to the 3′-end of the cDNA encoding human bFGF (Figure 1B). Briefly, HindIII restriction sites were introduced into the 3′-end of human RNase 1 or des.1-7RNase 1 cDNA and the 5′-end of human bFGF cDNA by site-directed mutagenesis. Two cDNAs coding for RNase–FGF fused proteins were inserted into the T7 expression vector pET-3a (Studier and Moffat, 1986).

**Expression and purification of RNase–FGF fused proteins**

For the expression of the two fused proteins, RNase 1 and bFGF (RNF) and des.1-7RNase 1 and bFGF (des.1-7RNF), E.coli BL21(DE3)pLyS5 (Studier and Moffat, 1986) and E.coli MM294(DE3)pLyS5 (Watanabe et al., 1990) were transformed with the plasmids described above. Each transformant was grown in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (10 µg/ml) overnight at 37°C. The culture was then diluted 50-fold with LB medium and further incubated at 37°C in an incubator shaker. When an $A_{600}$ of 0.6 was reached, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the growth was continued for a further 3 h at 37°C. Cells were harvested by centrifugation and washed once with physiological salt solution, and stored at −80°C until they were used.

**Fig. 1.** The schematic structures of RNase–FGF fused proteins. (A) The predicted three-dimensional structure of human RNase 1 with an extent of four amino acids adapted to the three-dimensional structure of bovine RNase A (Protein Data Bank entry 9RAT) is fused to the N-terminus of human basic FGF (Protein Data Bank entry 1BLA). The extended region in the C-terminus of human RNase 1 is shown as a dotted ribbon, and H12, K92, and H119 are the catalytic residues of RNase 1. This figure was produced using the RasMac molecular graphics program, version 2.6 (Sayle and Milner-White, 1995). (B) N-terminal, C-terminal amino acid residues and the linker regions of fused proteins are aligned to show the overall primary structures.
concentration from 0 to 2 M in 10 mM Tris--HCl, pH 7.5, for 60 min at a flow rate of 0.5 ml/min.

Induction of tyrosine phosphorylation
Tyrosine phosphorylation induced by RNase--FGF fused proteins was detected by an anti-phosphotyrosine antibody. Briefly, equal numbers of mouse BALB/c 3T3 A31 cells (Sasada et al., 1988) were plated onto 60-mm dishes and when confluence was reached, the cells were washed and starved in DMEM containing 0.2% CS for 2 days, and then a 1 nM concentration of bFGF, RNF, des.1-7RNF, RNase 1 or 10% CS was added. After 5 min, cells were washed once with ice-cold 20 mM HEPES--NaOH, pH 7.4, containing 150 mM NaCl, and harvested into 50 µl SDS-sample buffer. Samples were boiled for 3 min and 30 µl of the samples were electrophoresed on a 7.5% polyacrylamide gel, and then the proteins were transferred onto a nitrocellulose filter (0.45 µm; ADVANTEC, Japan). The blot was then blocked with 1% bovine serum albumin in TBS-T, the secondary antibody (goat anti-phosphotyrosine antibody clone PY20; ICN, USA) diluted moderately less active than RNase A (Seno et al., 1994). Furthermore, we have shown that truncation of seven amino acid residues in the N-terminal sequence of RNase 1 resulted in not only a much reduced activity, but also in a more pronounced reduction in the affinity to human placental RNase inhibitor (Futami et al., 1995). As for the bFGF molecule, the C-terminal structure significantly contributes to its affinity for heparin (Seno et al., 1990), while its N-terminal portion is flexible and not essential for its biological activity (Seno et al., 1989; Moy et al., 1996). Thus, we designed two fused proteins as immunotoxins based on human RNase 1 and human bFGF, as shown in Figure 1B, in which RNF is a protein fused between the C-terminus (Thr128→Ser) of RNase 1 and the N-terminus (Leu9) of the 147-amino acid form of human bFGF, and des.1-7RNF is a 7-N-terminal-residue truncated RNF. Figure 1A shows a schematic three-dimensional structure of RNase 1 and RNase A (Tilton et al., 1992) and residues 9–155 of bFGF (Moy et al., 1996) are linked with an arbitrary 4-residue linker.

Production, refolding, purification and characterization of RNase--FGF fused proteins
The fused proteins, RNF and des.1-7RNF, were expressed in E.coli strains MM294(DE3)pLysS and BL21(DE3)pLysS. Both strains gave almost the same results. In the case of RNF expression (Figure 2), induction with IPTG led to the accumulation of recombinant proteins up to about 50% of total cellular protein (Figure 2, lane 2). After lysis, the fused proteins were recovered as inclusion bodies, which were exhaustively washed to give a protein purity of about 90% (Figure 2, lane 3).

The proteins were dissolved in 6 M GdmCl, reduced with 2-mercaptoethanol, rapidly diluted in redox buffer, pH 8.5, containing 0.4 M GdmCl, and kept at 4°C overnight for refolding by SH--SS interchange. Utilizing the specific affinity of bFGF towards heparin (Seno et al., 1988), refolded fused proteins were purified by heparin affinity chromatography with the stepwise elution of NaCl. As shown in Figure 2, lanes 4 and 5, both RNF and des.1-7RNF thus purified gave almost single bands on SDS--PAGE. Analysis by reverse phase HPLC and cation-exchange HPLC also showed that they were composed of almost single species, respectively (data not shown). The yields of the fused proteins from a 1 l culture were both about 22 mg.

The amino acid composition and the N-terminal sequence of each RNF and des.1-7RNF were both confirmed to be coincident with the design. An N-terminal Met residue of
Fig. 2. SDS–PAGE of recombinant proteins. Samples were analyzed by 12.5% SDS–PAGE under reducing conditions. Lane 1, total cellular proteins of *E. coli* expressing RNF without induction by IPTG; lane 2, total cellular proteins of *E. coli* expressing RNF with induction by IPTG; lane 3, insoluble fraction of cell lysates; lane 4, purified RNF; lane 5, purified des.1-7RNF. The gel was stained with Coomassie brilliant blue R250.

Each fused protein takes the correct conformation to bind FGF receptors (Figure 3).

The results described above indicate that both the RNase and FGF domains in the RNase–FGF fused proteins well conserve the structure and function of the original proteins, respectively.

Effect of RNase–FGF on cell growth

A number of studies have suggested that bFGF may be an important autocrine growth factor for melanoma cells (Ahmed et al., 1997). Since the mouse metastatic melanoma B16/BL6 cell expresses both bFGF and high-affinity FGF receptor (Blanckaert et al., 1993), we employed this cell line expressing FGF receptor at high levels as a model target of the RNase–FGF fused proteins. Both of the RNase–FGF fused proteins showed similar dose-dependent growth inhibitory effects on B16/BL6 cells in the micromolar range (Figure 4). However, these growth inhibitory effects were not observed when cells were treated with an equimolar mixture of RNase 1 and bFGF.

Cytotoxic assays for human carcinoma-derived cell lines were also performed by systematic screening by the Cancer Chemotherapy Center, Japan. Table II lists seven sensitive cell lines against RNase out of 38 kinds of cell lines employed in this screening. Cell lines expressing FGF receptors (MDA-MB-231, MCF-7 and HT-29) were inhibited in their growth in the micromolar range. In contrast, no growth inhibition was observed on the human epidermoid carcinoma cell line A431, which does not express a FGF receptor, suggesting that RNase–FGF–fused proteins are properly folded and their RNase activity decreased due to the introduction of the FGF domain at the C-terminus. Des.1-7RNF was 20-fold less active than RNF.

Analysis by heparin affinity HPLC under the conditions of the linear gradient elution of NaCl (0–2 M) at pH 7.5 indicated that RNF and des.1-7RNF were eluted at 1.65 and 1.60 M NaCl, while bFGF and RNase 1 were eluted at 1.30 and 0.62 M NaCl, respectively. These results suggest that the FGF domain is also properly folded in each fused protein to maintain the high affinity towards heparin.

The potential of the fused proteins to induce tyrosine phosphorylation of some cellular proteins including the major substrate of p92-95 (FGF receptor substrate 2; FRS2), which is specific to the stimulation of the FGF-receptor mediated Ras/MAPK signaling pathway (Kouhara et al., 1997), was assessed. RNF and des.1-7RNF induced the tyrosine phosphorylation of bFGF, indicating that the FGF domain in each fused protein takes the correct conformation to bind FGF receptors (Figure 3).

The results described above indicate that both the RNase and FGF domains in the RNase–FGF fused proteins well conserve the structure and function of the original proteins, respectively.

**Table I. Relative RNase activity of recombinant proteins**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase 1</td>
<td>100</td>
</tr>
<tr>
<td>RNF</td>
<td>9.7</td>
</tr>
<tr>
<td>des.1-7hRNase</td>
<td>13.3</td>
</tr>
<tr>
<td>des.1-7RNF</td>
<td>0.66</td>
</tr>
</tbody>
</table>

**Table II. Relative RNase activity of recombinant proteins**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase 1</td>
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</tr>
<tr>
<td>RNF</td>
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</tr>
<tr>
<td>des.1-7hRNase</td>
<td>13.3</td>
</tr>
<tr>
<td>des.1-7RNF</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Assay conditions are described in Materials and methods.
Fig. 4. Growth inhibitory effects of RNase–FGF fused proteins on mouse melanoma B16/BL6 cells. Mouse melanoma B16/BL6 cells (500 cells/well) were cultured for 2 days with RNF (●), des.1–7RNF (○) or equimolar amounts of RNase 1 and bFGF (△). Cell growth of each well was monitored by MTT assay. MTT formazan formed in the cells was solubilized with SDS solution, absorbance at 550 nm was measured and the percent growth against the cells treated with media alone was calculated and plotted. Each point and vertical line show the mean value and the standard deviation of triplicates, respectively.

Table II. Specific growth inhibition of human carcinoma-derived cells by RNF

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>GI_{50}a (µM)</th>
<th>FGF receptor (sites/cell)</th>
<th>High affinity/Low affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Breast carcinoma</td>
<td>1.7</td>
<td>8.5 × 10^{3}/2 × 10^{4}b</td>
<td>High affinity/Low affinity</td>
</tr>
<tr>
<td>SF-539</td>
<td>Glioma</td>
<td>1.7</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>BSY-1</td>
<td>Breast carcinoma</td>
<td>2.2</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>OVCA-5</td>
<td>Ovarian carcinoma</td>
<td>3.6</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>KM-12</td>
<td>Colon carcinoma</td>
<td>3.8</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon carcinoma</td>
<td>4.8</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast carcinoma</td>
<td>6.4</td>
<td>1.75 × 10^{3}/NDc</td>
<td></td>
</tr>
<tr>
<td>A431</td>
<td>Epidermal carcinoma</td>
<td>&gt;10</td>
<td>NDc</td>
<td></td>
</tr>
</tbody>
</table>

aGI_{50} indicates the concentration of RNF that exhibits 50% attenuation of cell growth after 2 days of treatment.
bFrom Peyrat et al. (1991)
cFrom Beitz et al. (1992)
ND, not detected.

Discussion

FGF receptors constitute a family of heterogeneous proteins, many of which are expressed in solid tumors, and several of which were shown to be targeted using FGF as a ligand (Lappi, 1995). Since the FGF molecule shows a specific affinity to a wide range of FGF receptor proteins, FGF would be a better ligand than antibodies specific to single species of FGF receptor. On the other hand, human pancreatic RNase 1 has the potential to be engineered into a toxin. The aim of this study was the preparation of agents targeting FGF receptors...
overexpressed in tumors by designing a fusion protein between human RNase 1 and human bFGF.

We designed two RNase–FGF fusion proteins, RNase and des.1-7RNase, in which the C-terminal Ser128 of human RNase 1 or des.1-7RNase 1 was fused to the Leu9 of human bFGF (Figure 1), which were expressed in E.coli as inclusion bodies. Since the expression of RNase activity would inhibit host cell growth, formation of inclusion bodies should be advantageous. In this case, however, the folding into the active conformation becomes a crucial step. Nevertheless, we have obtained good yields of folded RNase (or des.1-7RNase) from inclusion bodies by SH–SS interchange reaction in redox buffer (about 22 mg from a 1 l culture). One of the critical points to emerge was the use of a rather high concentration (0.4 M) of GdmCl in the folding. The presence of 0.4 M salt, such as NaCl, KCl, NH4Cl or Arg–HCl, also gave good yields of the folded protein. At concentrations below 0.2 M, besides the increase in the formation of precipitates, several improperly folded species were obtained. The latter was judged from the lower affinity on a heparin column and from the elution profile of multiple peaks on a cation-exchange HPLC analysis (unpublished data). Because highly purified fused protein was efficiently folded into a single species in a salt concentration independent manner, an electrostatic interaction between the fused protein and the other materials in the inclusion bodies, such as nucleic acids, may interfere with the correct folding of the fused proteins (unpublished data).

Folded RNase and des.1-7RNase were purified using a one-step procedure by heparin affinity chromatography. RNase and des.1-7RNase, thus purified, retained well the physico-chemical and biological properties originating from RNase, des.1-7RNase and bFGF. The RNase activity was decreased 8–15-fold (Table I), probably due to the interdomain steric hindrance rather than incorrect folding, because the stability of each domain against trypsin digestion was similar to those of the unfused proteins, respectively (data not shown).

Both RNase and des.1-7RNase showed dose-dependent growth inhibitory effects on mouse metastatic melanoma B16/BL6 cells, which are known to overexpress FGF receptors, while no inhibition was observed in a mixture of RNase 1 and bFGF under the conditions employed (in the presence of 3% serum) (Figure 4). Furthermore, the fused proteins did not show any effect on normal fibroblasts such as BALB/c 3T3 (data not shown). This growth inhibitory effect of RNase and des.1-7RNase was shown to be specific to the cells overexpressing FGF receptors, as expected. RNase and des.1-7RNase did not kill B16/BL6 cells but just slowed down the speed of cell growth (Figure 5). However, the cells were not arrested at any particular stage in the cell cycle when treated with these fused proteins (Figure 6). Similar results have been described in the cell growth suppression of onconase, a member of the pancreatic-type RNase family isolated from frog oocytes (Mikulski et al., 1992). Therefore, a prolonged cell cycle may be a common property of RNase-mediated growth inhibition. We also examined apoptosis assays on B16/BL6 cells treated with 5 μM RNase by electrophoresis of intermemosomal DNA; however, no DNA fragmentation was observed. In this context, the RNase–FGF fused proteins prepared here were cytostatic rather than cytotoxic.

RNase also showed a growth inhibitory effect on the growth of many carcinoma-derived human cell lines (Table II). MDA-MB-231, HT-29 and MCF-7 are known to overexpress FGF receptors (Peyrat et al., 1991; Murgue et al., 1994), while the others have not yet been examined in this respect. We studied the relationship between GI50s by RNase and FGF receptor levels in MDA-MB-231 and MCF-7, which suggested the existence of two possible pathways for internalization of bFGF: high affinity receptor-mediated and low affinity receptor (heparin-like molecule)-mediated, also described previously (Rognoni and Moscatelli, 1992). These results again suggest that the cytostatic effect of the RNase–FGF fused proteins is specific to the cells overexpressing FGF receptors.

Basic FGF has been suggested to be involved in a number of proliferative diseases and pathogenesis (Baird et al., 1986), including angiogenesis, which is important in carcinogenesis (Folkman et al., 1989). Expression of bFGF specific receptors (FGFR1) are also up-regulated in the mesangial proliferative stage of glomerular failure (Jyo, 1996). All the cells pathologically associated with these disorders should be potential targets of a bFGF-based growth inhibitor. In the case of the proliferative disorders, such as glomerulonephritis, it may be important to suppress only the proliferation of cells without killing them. The cytostatic nature of RNase or des.1-7RNase may be useful for the treatment of such bFGF-induced proliferative disorders.

Some members of pancreatic-type RNase family, such as onconase and bovine seminal dimeric-RNase (BS-RNase), have cytotoxic activity against cancer cells and/or anti-HIV activity (Vescia et al., 1980; Wu et al., 1993; Saxena et al., 1996). Onconase is now in clinical trials for cancer therapy (Mikulski et al., 1993). All investigated mammalian pancreatic-type RNases, except for BS-RNase, are known to be severely inhibited by a RNase inhibitor that occurs in the cytoplasm of virtually all mammalian cells (Futami et al., 1997; Hofsteenge et al., 1998). Both onconase and BS-RNase have also been reported to weakly interact with RNase inhibitor (Murthy and Sirdeshmukh, 1992; Wu et al., 1993).

Hence, the first mechanism of cytotoxicity of these RNases has been explained as follows. Cytotoxic RNases are adsorbed on the cell surface and internalized into cells. Second, they degrade cellular RNA evading cytosolic RNase inhibitor and inhibit protein synthesis as a result (Wu et al., 1993; Kim et al., 1995). The mechanism proposed above explains why the cytotoxic potentials of the present RNase–FGF fusion proteins are not so high. RNase–FGF fused proteins are considered to be effectively internalized into the cytosol through bFGF receptors. Because the cytotoxic potency of an RNase should correlate with the remaining RNase activity in the presence of RNase inhibitor in the cytosol, and because cytosolic RNase inhibitor should severely inhibit the activity of human RNases, the cytotoxic effect of RNase becomes moderate. In this context, des.1-7RNase is an interesting molecule because des.1-7RNase shows a lower affinity than RNase 1 to RNase inhibitor (Futami et al., 1995). However, the growth inhibitory effect of des.1-7RNase was almost identical to that of RNase (Figures 4 and 5). Because the RNase activity of des.1-7RNase was 20-fold lower than that of RNase, the effect of decreased affinity on the RNase inhibitor may be compensated by the decreased RNase activity. Furthermore, the N-terminally truncated RNase domain may be unstable against proteolysis in the cytosol. This effect may also compensate for the effect of the decreased affinity to the RNase inhibitor. If we could internalize more RNF molecules relative to the level of cytosolic RNase inhibitor, a much stronger cytotoxicity would result. The balance between the rate of internalization through FGF receptors and the rate of proteolytic degradation may determine the concentration of...
RNase in the cytosol. Since the number of FGF receptors on a cell surface would limit the former rate, we could only change the latter rate by stabilization of the RNase domain against proteolysis by means of protein engineering. Thus, in order to potentiate the cytotoxicity of RNase, we need to design the RNase domain with a lower affinity to RNase inhibitor, high RNase activity and sufficient stability. We could successfully demonstrate by the RNase–FGF fused proteins as humanized immunotoxin of cell-type specific growth inhibitors.

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Reference


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Human RNase–FGF: a humanized immunotoxin