Insertional-fusion of basic fibroblast growth factor endowed ribonuclease 1 with enhanced cytotoxicity by steric blockade of inhibitor interaction

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Abstract Basic fibroblast growth factor (bFGF) was inserted in the middle of human ribonuclease 1 (RNase1) sequence at an RNase inhibitor (RI)-binding site (Gly89) by a new gene fusion technique, insertional-fusion. The resultant insertional-fusion protein (CL-RFN89) was active both as bFGF and as RNase. Furthermore, it acquired an additional ability of evading RI through steric blockade of RI-binding caused by fused bFGF domain. As a result, CL-RFN89 showed stronger growth inhibition on B16/BL6 melanoma cells than an RI-sensitive tandem fusion protein. Thus, the insertional-fusion technique increases accessible positions for gene fusion on RNase, resulting in construction of a potent cytotoxic RNase.

Keywords: Ribonuclease; Basic fibroblast growth factor; Insertional-fusion protein; Targeting; Protein engineering

1. Introduction

Several kinds of hybrid proteins have been constructed to produce bifunctional proteins and utilized as tools such as detectors for biological molecules. Generally, those hybrid proteins have been built by end-to-end fusion (tandem-fusion) of two genes of component proteins. The alternative is to insert the second protein (the insert protein) into the middle of the sequence of the host protein in-frame [1,2]. This new mode of gene fusion (insertional-fusion) is expected to give an additional configuration between two component domains and to make it possible to create the hybrid protein with the ideal 3D structure for new functions. However, the insertional-fusion has not been popular because it is apparently more limited and requires precise information on the parental protein structures. Therefore, insertional-fusion has first been achieved by random screening to find accessible site for insert sequences [3]. Recently, it has been suggested that the tolerance of protein structures to very large protein insertions is more general than previously thought [4], and some insertional-fusion proteins have been designed based on the 3D structures of component proteins and successfully obtained in active structures. However, the resultant proteins were less stable than the parental proteins (reviewed in [2]).

We used this insertional-fusion technique for engineering human RNase1 to be cytotoxic. RNase1 itself is not cytotoxic as a majority of RNase superfamily. The major reasons considered for it are (1) the neutralization of their ribonucleolytic activity by RNase inhibitor (RI) expressed ubiquitously in the cytosol [5], and (2) insufficient binding to the target cells. Therefore, engineered variants of RNase1, which could efficiently reach the cytosol of the target cells [6,7] and/or to maintain their activity there by evading the action of RI [8], were reported to acquire cytotoxicity. Previously, we constructed cytotoxic RNase by fusing human basic fibroblast growth factor (bFGF) to the C-terminus of RNase1 [9]. The resultant tandem-fusion protein (RNF) could inhibit the growth of malignant cells with high levels of cell surface FGF receptor. However, its activity was still weak (IC_{50} value was more than 1 μM) probably because it could not evade inactivation by RI.

In this study, bFGF was inserted into RNase1 at the exact RI-binding site that is in the middle of RNase sequence. The resultant insertional-fusion protein had both abilities of efficient binding to target cells and evading RI by masking the RI interaction site with the targeting protein of bFGF. Furthermore, an additional intramolecular disulfide-bridge was introduced in the insertional-fusion protein to increase its conformational stability that was suggested as another important determinant of RNase-mediated cytotoxicity [10,11]. These insertional-fusion proteins were evaluated for activities of both RNase and bFGF, for stability against protease digestion, and for growth inhibition on malignant cells.

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Abbreviations: bFGF, basic fibroblast growth factor; CL-RNase1, 4-118 cross-linked RNase1 mutant; RFNs, insertional-fusion proteins between hRNase1 and bFGF; CL-RFNs, insertional-fusion proteins between CL-RNase1 and bFGF; RI, ribonuclease inhibitor

2. Materials and methods

2.1. Materials

Recombinant human RNase1, 4–118 cross-linked RNase 1 (CL-RNase 1) and human bFGF (147 amino acid form) were purified from *Escherichia coli* as described previously [9,12].

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2.2. Construction of insertional-fusion proteins

A cDNA encoding bFGF (19–146) (N-terminal 18 residue-truncated form of bFGF) was amplified by polymerase chain reaction using primers (5′-TTTCCGCGGGCAGCGACCCCAAGCGGCTGTAC-3′) and 5′-ATTCCGCGGAGCTTTCAGCAGACATTGG-3′) and pBO126 [9] as a template. On the other hand, a *Sac*II site was introduced at the position of Pro19 or Gly89 of RNase1 cDNA into an RNase1 expression vector of pBO26 [13] by site-directed mutagenesis. The resultant plasmids were cleaved with *Sac*II and ligated in-frame with the *Sac*II fragment of bFGF (19–146) to construct the expression vectors for insertional-fusion proteins with bFGF insertion at Pro19 (RFN19) or at Gly89 (RFN89) of RNase1, respectively. Similarly, the expression vectors for fusion proteins with bFGF (19–146) insertion at Pro19 (CL-RFN19) or at Gly89 (CL-RFN89) of CL-RNase1 were constructed using an expression vector for CL-RNase1, pBO383 [12], instead of pBO26. The expression vector for an insertional-fusion protein with bFGF (21–144) insertion at Gly89 of CL-RNase1 (CL-RFN89-2) was also constructed by insertion of the bFGF (21–144) fragment amplified with primers (5′-ATACCGCGGCCAAGCG-GCTGTAC-3′ and 5′-AGCCCGCGGCAGACATTGGAAG-3′). The schematic structures of these RFNs and CL-RFNs are depicted in Fig. 1A.

2.3. Expression, refolding and purification of insertional-fusion proteins

All of the insertional-fusion and tandem-fusion proteins were expressed as inclusion bodies in *E. coli*, and then solubilized, refolded, and purified by the same procedure as described previously [13,14]. The purified proteins were concentrated by ultrafiltration with Ultrafree-4 centrifugal filter (Biomax-5K NMWL, Millipore, USA). Protein concentrations were determined by UV spectroscopy as described [15].

2.4. Assays for RNase activity

Ribonucleolytic activity of the insertional-fusion proteins on yeast transfer RNA was measured as described previously [16]. To evaluate the affinity of RI for each protein, their RNase activity was measured in the presence and absence of 130 units/ml of recombinant human placental RI (Wako, Japan) (1 unit of RI is that amount required to inhibit 50% the activity of 5 ng of RNase A) and 1 mM DTT.

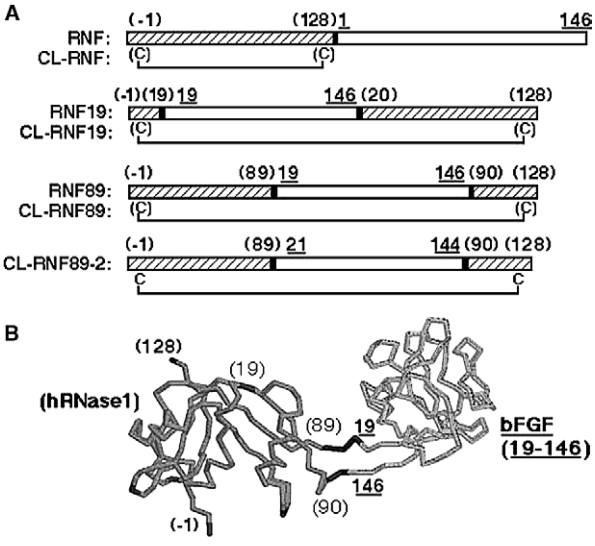

Fig. 1. The schematic structures of insertional-fusion proteins between bFGF and RNase1. (A) The bFGF (white bar) was inserted in host RNase1 (striped bar) by gene fusion. Black bars indicate linker sequences derived from restriction sites for insertion. The positions of additional disulfide cross-link and introduced cystein residues are shown. (B) Model of CL-RFN89. The structure derived from RNase1 (PDB No. 1UFS, unpublished data) is dark gray and that from bFGF (PDB No. 2FGF [27]) is light gray. This figure was produced using the RasMac molecular graphics program, version 2.6 [28]. The amino acid residue numbers derived from RNase1 sequence are indicated in parenthesis and those from bFGF are underlined. Extra methionine residues at −1 positions of these proteins derived from the initiation codons were all confirmed by N-terminal amino acid sequence analysis, and indicated as (−1).

2.5. Mitogenic activity toward serum-starved murine fibroblasts

Mouse Balb/c 3T3 A31 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui, Japan) containing 10% fetal bovine serum (FBS). The cells were plated onto 96-well plates (1.5×10^3 cells/well) and cultured for 12h. The medium was then replaced with DMEM containing 0.5% FBS (100 μl) and the cells were incubated for 1 day to starve the cells. Then, 100 μl of samples was added to the wells. After 2 days of cultivation, the cell growth was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously [17].

2.6. Assays for growth inhibitory effect

Cytotoxicity on mouse metastatic melanoma B16/BL6 cells was evaluated as described previously [9].

2.7. Tryptic digestion

Digestion of each protein was carried out under the physiological conditions with trypsin [12]. Five μg of proteins dissolved in 15 μl of 75 mM Tris–HCl, pH 8.0, was incubated with indicated concentrations of TPCK–trypsin at 37 °C for 30 min. The reactions were stopped by addition of a sample buffer and analyzed by SDS–PAGE on 15% polyacrylamide gel under reducing conditions.

3. Results and discussion

3.1. Design of insertional-fusion proteins between RNase1 and bFGF

Previous studies on native cytotoxic RNases as well as engineered cytotoxic RNases have shown that the mechanism of RNase-mediated cytotoxicity consists of two steps: binding to the target cells to reach cytosol and catalytic cleavage of cellular RNA. If a targeting protein can be fused at the exact site of RI-binding on the RNase1 molecule, the resultant fusion protein is expected to show cytotoxicity through both abilities of efficient cell binding and efficient ribonucleolytic activity in the cytosol in which RI is ubiquitously expressed. However, it is impossible to construct such a structure by the conventional tandem-fusion technique, because both termini of RNase are apart from the RI-binding sites. Previously, Suzuki et al. conjugated a targeting protein (transferrin) with RNase1 at the RI-binding site via a thioether bond [18] with an aim similar to ours and successfully obtained a cytotoxic RNase. In this study, we used insertional-fusion technique to place a targeting protein at the RI-binding site. On the design of insertional-fusion proteins, we selected bFGF as a targeting protein for insertion, since the N- and C-termini of "beta-trefoil core region (residues 19–146)" of bFGF are near each other (5.3 Å between Cαs of Asp19 and Lys145) as shown in Fig. 1B. This fragment has been shown to be sufficient for its biological activities [19]. On the other hand, an RI-binding site of Gly89 [20] was selected as the insertion site on RNase1. Pro19 irrelevant to RI-binding was also selected as another insertion site for control. Taken together, we constructed two insertional-fusion proteins of RFN89 and RFN19, respectively (Fig. 1A). Furthermore, we constructed two additional insertional-fusion proteins named CL-RFN89 and CL-RFN19 using a stabilized mutant of RNase1 (CL-RNase1) as a host, to obtain more stable insertional-fusion proteins. In CL-RNase1, two cystein residues introduced at positions 4 and 118 of RNase1 form an additional intramolecular disulfide cross-link to stabilize its structure [12]. Furthermore, CL-RFN89-2 was constructed by inserting bFGF

(21–144) sequence at Gly89 of CL-RNase1 to shorten linker sequences.

3.2. Activities of RNase domain and bFGF domain of insertional-fusion proteins

When these insertional-fusion proteins were expressed directly in *E. coli*, they were produced as inclusion bodies. After refolding, the yields of all the insertional-fusion proteins purified from 1 liter-culture were about the same, from 5 to 10 mg. As shown in Table 1, all of these insertional-fusion proteins were ribonucleolytically active (30% or more of wild-type RNase1). The results indicate that their RNase domains were properly folded as the parental RNase1, nevertheless their primary sequences are divided and intervened by long bFGF sequence.

To access their actual activity under cytosolic conditions, RNase activity was measured in the presence of RI (Fig. 2). RNase activity of RFN19, CL-RFN19, and RNF was completely inhibited by RI as RNase1 was. In contrast, RFN89, CL-RFN89, and CL-RFN89-2 retained their activity by 85% or more even in the presence of 200-fold molar excess amount of RI. From these results, the values of inhibitor constant (K_i) of RFN89, CL-RFN89 and CL-RFN89-2 to RI were estimated to be approximately 100–200 nM (Table 1), which were 10^4-fold higher than that of wild-type RNase1 ($K_i = 20$ pM [21]). Thus, the insertional-fusion proteins with bFGF insertion at the RI-binding site of RNase1 acquired the ability of evading RI.

Mitogenic activity of these insertional-fusions was accessed to evaluate the activity of bFGF domain (Table 1). All the insertional-fusion proteins showed strong mitogenic activity as much as the tandem RNF. Thus, the bFGF domain is also properly folded in the middle of RNase1 sequence to bind cell-surface FGF-receptors.

3.3. Stability of insertional-fusion proteins against tryptic digestion

The conformational stability of RNases was suggested as another important factor for their cytotoxic activity [10,11]. However, in general, ''insertional-fusion proteins'' have been shown to be less stable than the parental proteins [4]. In the case of another insertional-fusion protein between SH3 domain and protein L, the free energy for unfolding of SH3 was

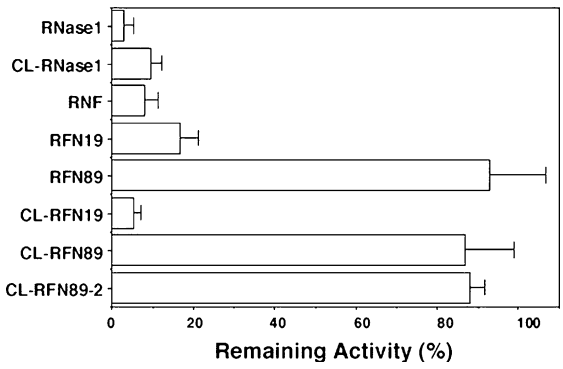

Fig. 2. Enzymatic activity of the insertional-fusion proteins in the presence of RI. Inhibition of ribonucleolytic activity of the insertional-fusion proteins by RI was assayed with tRNA as a substrate. Remaining activity (%) is indicated as relative activity in the presence of RI (a molar ratio of enzyme to inhibitor of 1:200–300) to that in the absence of RI. The values were means ± S.D. of three experiments.

reported to be reduced by 1.2 kcal/mol [22]. We therefore evaluated the stability of the insertional-fusion proteins by tryptic digestion. As shown in Fig. 3, the insertional-fusion proteins were digested faster than the tandem-RNF and parental RNase1, indicating that their structures are considerably destabilized. RFN89 was less stable than RFN19 in spite of same length and sequence of the insert, suggesting that some significant destabilization such as conformational strain might be associated with RFN89. Thus, although RFN89 was obtained as active form, it was considered to be too unstable to resist proteolytic digestion systems in the target cells. CL-RFNs, in which an additional intramolecular disulfide-bridge was introduced to stabilize their structure, were more stable than the respective RFNs, although they were still less stable than the tandem-RNF. As another attempt, both of the spacer sequences between host and insert domains were shortened by two residues in CL-RFN89-2 (Fig. 1A), however, obvious additional stabilization or destabilization was not observed in the tryptic digestion assay (data not shown). Similar results were obtained by using proteinase K instead of trypsin, suggesting that the increased protease-sensitivity was not sequence specific but resulted from the decreased conformational stability of these fusion proteins because protease digestion of small globular proteins generally occurs from the unfolded

Table 1
Characteristic properties of the insertional-fusion proteins between RNase1 and bFGF

Proteins	RNase activity (%)[a]	Inhibitor constant K_i (nM)[b]	Mitogenic activity ED_{50} (pM)[c]	Growth inhibition IC_{50} (μM)[d]
RNF	84.8 ± 3.5	2.1 ± 0.8	18.6 ± 16.5	1.6 ± 0.8
RFN19	50.2 ± 3.2	4.7 ± 1.4	9.5 ± 6.8	1.5 ± 1.1
RFN89	68.2 ± 9.1	1.3 ± 0.3	26.0 ± 15.3	0.87 ± 0.30
CL-RFN19	76.0 ± 7.4	118 ± 9.8	8.7 ± 5.4	>3
CL-RFN89	36.8 ± 4.6	110 ± 51	6.7 ± 3.0	0.32 ± 0.14
CL-RFN89-2	33.1 ± 1.5	193 ± 34	5.5 ± 4.0	0.23 ± 0.17
HRNase1	100	<1	n.d.[e]	n.d.[e]
CL-RNase	38.9 ± 1.8	1.8 ± 0.8	n.d.[e]	n.d.[e]
bFGF	–	–	118 ± 85	>3

[a] Ribonucleolytic activity was measured at 37 °C by using yeast transfer RNA as a substrate and expressed as relative activity (%) to that of RNase1.
[b] Inhibitor constant to RI was calculated from the data shown in Fig. 2, as described previously [16].
[c] Mouse fibroblast Balb/c 3T3 A31 cells were cultured with various proteins under low-serum (0.5%) conditions. After 3 days, the cell growth was measured by the MTT assay.
[d] Mouse B16/BL6 melanoma cells were cultured with various proteins for 3 days. The growth of the cells was measured by the MTT assay.
[e] Not detected.

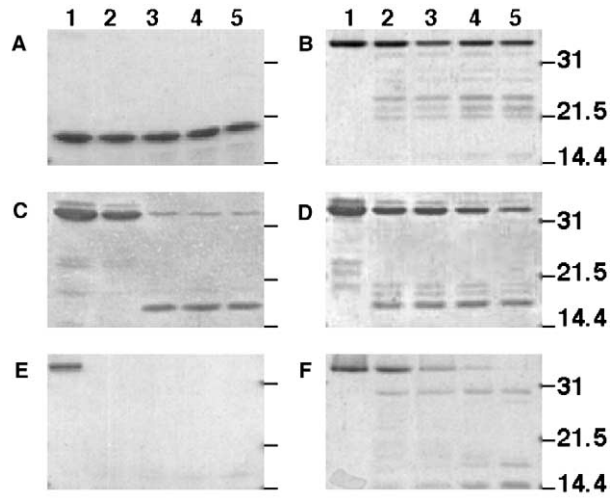

Fig. 3. SDS–PAGE analysis of digestion of the insertional-fusion proteins with trypsin under physiological conditions. The insertional-fusion proteins were incubated in the presence of various concentrations of trypsin at pH 8.0 and 37 °C for 30 min, and then subjected to SDS–PAGE. The concentrations of trypsin were 0 μg/ml (lane 1), 0.5 μg/ml (lane2), 1.0 μg/ml (lane 3), 2.1μg/ml (lane 4), and 4.2 μg/ml (lane 5), respectively. Panels A, hRNase1; B, RNF; C, RFN19; D, CL-RFN19; E, RFN89; F, CL-RFN89, respectively. Bars on the right show the positions of some molecular protein size markers.

state in the unfolding equilibrium as described previously [23,24]. The results from the N-terminal sequence analysis of the partially digested fragments of the RFNs indicated that the host domain was much less stable than the insert domain and thus the digestion occurred preferentially in the host domain (data not shown). This suggested that the host domain (RNase) was preferentially destabilized in the RFNs. Although the insert domain was also destabilized to be digested faster than bFGF itself, the effect to the host domain is considered more significant than the insert domain. Considering the unstable nature of RFN89, RNase1 is thought to have only enough conformational stability (5.7 kcal/mol [12]) to tolerate insertional-fusion. On the other hand, when an intramolecular disulfide-bridge was introduced in RNase1, the conformational stability of the resultant mutant of CL-RNase1 increased by approximately 2 kcal/mol [12]. These observations suggest that introduction of additional intramolecular disulfide bridges would be a general and effective strategy against destabilization associated with insertional-fusion.

3.4. Growth inhibition by insertional-fusion proteins

All of the insertional-fusion proteins except CL-RFN19 inhibited the growth of B16/BL6 melanoma cells similar to the tandem-fusion protein RNF (Table 1). As expected, the activity of the insertional-fusion proteins with higher stability and RI-evading ability (CL-RFN89 and CL-RFN89-2) was 5- and 7-fold stronger than that of RNF, respectively. In contrast, RI-sensitive CL-RFN19 showed little growth inhibitory effect, although it was more stable and more ribonucleolytically active in the absence of RI than RI-resistant ones.

In this study, we measured four factors possibly involved in the growth inhibitory effect of the fusion proteins between RNase1 and bFGF: RNase activity itself derived from RNase domain, that in the presence of RI, the mitogenic activity derived from bFGF domain, and the stability against proteases. Among them, the RNase activity in the presence of RI as a result of the RI-evading ability was indicated as the most important factor for the growth inhibition. The facts that the difference in the inhibitor constant (K_i) values among the fusion proteins (more than 100 nM for RI-evading CL-RFN89s and less than 5 nM for other RI-sensitive fusion proteins) was remarkable and the value was relatively close to their IC50 values might be the reasons for the effectiveness (Table 1). In addition, the structural stability was also suggested to affect the growth inhibitory activity since the increase in growth inhibitory activity of the least stable RFN89 was only slight, although it showed highest RNase activity in the presence of RI. These results suggested that the effect of enhanced RNase activity in the presence of RI was almost completely cancelled by the rapid decrease of the actual concentration of active RFN89 in the cells caused by the rapid degradation of unstable RFN89. In contrast, intrinsic RNase activity is considered to have little effect on the present growth inhibition, because the enhancement of RI-evading was more prominent than the decrease in RNase activity of the fusion proteins (Table 1 and Fig. 2). As for the targeting ability derived from bFGF, the lower ED50 values in the mitogenic activity of the fusion proteins suggested their higher affinity to high affinity tyrosine-kinase receptors for bFGF but their similar elution profile on heparin-column HPLC (data not shown) suggested that their affinity to low-affinity receptors would be equivalent to that of bFGF. However, this targeting ability also had little effect on the growth inhibition. The ED_{50} values of mitogenic activity (from 0.005 to 0.03 nM) as well as reported affinity of bFGF to their cell surface receptors (K_d of 0.2 nM for high-affinity receptors and 9 nM for low-affinity receptors [25]) were extremely lower than the IC_{50} values of their growth inhibition

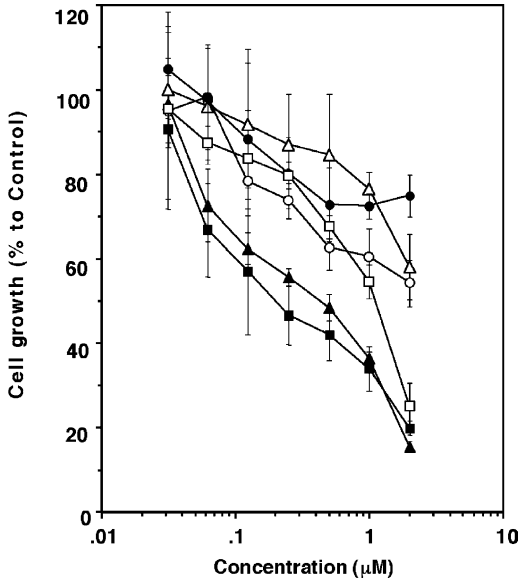

Fig. 4. Growth inhibitory effects of the insertional fusion proteins on mouse melanoma B16/BL6 cells. B16/BL6 cells (5×10^2 cells/well) were cultured for 3 days with RNF (open triangle), RFN19 (open circle), RFN89 (open square), CL-RFN19 (solid circle), CL-RFN89 (solid triangle), and CL-RFN89-2 (solid square), respectively. Cell growth of each well was monitored by MTT assay. Each point and vertical line show the mean value and the S.D. of triplicates, respectively.

(more than 200 nM), suggesting that all of the fusion proteins already had sufficient affinity for their receptors (see Fig. 4).

Considering CL-RFN89 as an anti-cancer cytotoxin, use of growth factors such as bFGF as a targeting carrier may be controversial, because it might be possible that their mitogenic effect could cancel their growth inhibitory effect or cause the proliferation of the target cancer and/or the normal cells in vivo. Although little mitogenic effect was observed on Balb/c 3T3 cells as well as B16/BL6 melanoma cells in the normal culture containing 10% serum (data not shown), this possibility of CL-RFN89 should be checked by using the individual target cancer cells as well as normal tissues before therapeutic application. This problem will be solved by using antagonistic bFGF mutants that possess decreased mitogenicity with little change in affinity for cell surface receptors [26]. Since CL-RFN89 also inhibits the proliferation of other FGFR-positive cells such as human vein endothelial cells, the possibility of its therapeutic application to neovascular diseases is now under investigation.

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