Cancer Chemother Pharmacol (2003) 52: 270–276
DOI 10.1007/s00280-003-0666-0

ORIGINAL ARTICLE

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Targeting of a novel fusion protein containing methioninase to the urokinase receptor to inhibit breast cancer cell migration and proliferation

Received: 30 December 2002 / Accepted: 30 April 2003 / Published online: 25 June 2003

Abstract It has been shown that methionine depletion inhibits tumor cell growth and reduces tumor cell survival. A novel fusion protein targeted specifically to tumor cells was developed. The fusion protein contained two components: the amino terminal fragment of human urokinase (amino acids 1–49) that binds to the urokinase receptor protein expressed on the surface of invasive cancer cells, and the enzyme L-methioninase (containing 398 amino acids) which depletes methionine and arrests the growth of methionine-dependent tumors. The influence of the fusion protein on the growth and motility of human breast cancer cells was examined using a culture wounding assay. It was determined that MCF-7 breast cancer cells, used in this study, were methionine-dependent and that the fusion protein bound specifically to urokinase receptors of the surface of the cancer cells. Further treatment of the cancer cells with fusion protein over the concentration range 10^{-8} to 10^{-6} *M* produced a dose-dependent inhibition of both the migration and proliferation index of MCF-7 cells in the culture wounding assay over a period of 1 to 3 days. The results of this study suggest that this novel fusion protein may serve as a prototype for specific targeting of methioninase and perhaps other cytotoxic agents to cancer cells.

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Keywords Breast cancer · Methioninase · Urokinase · Cancer targeting · Fusion protein

Abbreviations *ATF* Amino-terminal fragment of urokinase (amino acids 1–49) · *PMSF* Phenylmethylsulfonyl fluoride · *SDS-PAGE* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis · *TPCK N-p*-Tosyl-L-phenylalanine chloromethyl ketone

Introduction

Results obtained over the past 40 years have demonstrated that tumor cells of all types tested have an elevated growth requirement for methionine compared to normal cells [1, 2]. Numerous lines of cancer cells are unable to survive and grow when the amino acid methionine is replaced in the medium with homocystine. However, normal adult cell lines survive and grow well with this substitution. For example, breast carcinosarcoma and lymphatic leukemia cells do not retain viability after 20 days in medium devoid of methionine but with added homocystine; on the other hand, normal liver fibroblasts, breast fibroblasts, and prostate fibroblasts grow normally under these same conditions [1]. Further studies have shown that methionine-dependent cells arrest in the G_2 and G_1 phases of the cell cycle and subsequently die at methionine concentrations less than 5 μM regardless of high concentrations of homocystine precursors and folates [3].

Subsequent to the tests of the effect of methionine on cancer and normal cells in cell culture, there have been in vivo tests of the effect of methionine depletion on cancer cells. One study was performed on mice with human brain tumor xenografts [4]. With a combination of dietary restriction of methionine, homocystine, and choline and synchronous treatments with intraperitoneal injections of L-methioninase and homocystine, tumor stasis was achieved in 100% of treated animals within 4 days

of treatment, and regression was seen in one-third of animals after a 10-day period. The methioninase was not toxic to the mice. A phase I clinical trial of the treatment of three breast cancer patients with L-methioninase showed no side effects, although a relatively long (10-h) period of i.v. infusion was needed to reduce serum L-methionine to very low levels [5].

The urokinase-type plasminogen activator (uPA) receptor has consistently been found to be present at the invasive foci of most human cancers [6]. Also known as urokinase, uPA appears to be the enzyme primarily responsible for the generation of plasmin during the process of extracellular matrix degradation. The ability of cancer cells to degrade extracellular matrices is critical to the metastasis of these cells. Urokinase consists of an A chain and a B chain, with the A chain responsible for binding to the receptor [7]. Further, studies have shown that residues 12–32 in the A chain are critical for binding to the receptor [8].

The major objective of this project was to specifically target L-methioninase to breast cancer cells by combining L-methioninase in a fusion protein that contained the amino terminal fragment of urokinase, which binds to the urokinase receptor expressed on the surface of the cancer cells. Our major hypothesis was that this fusion protein would bind specifically to urokinase receptors and produce selective cytotoxicity to methionine-dependent breast cancer cells.

Materials and methods

Plasmids and bacterial strains

A pKK223-3 plasmid containing the gene for L-methioninase (containing 398 amino acids and with a calculated molecular weight of 42.7 kDa) from *Pseudomonas putida* was kindly provided by Dr. Dennis Carson of the University of California, San Diego [9]. Plasmid pULB1221 containing the gene for human urokinase was kindly provided by Dr. Paul Jacobs of the Free University of Brussels, Belgium [10]. Plasmid pKK223-3, with the *tac* promoter and an ampicillin resistance gene, was obtained from Amersham Biosciences (Piscataway, N.J.). *Escherichia coli* JM105 was used as the host for both vector construction and protein expression.

Plasmid construction

The following fusion protein gene was constructed: N-(amino acids 1–49 of urokinase A chain)-Gly-Ser-Gly-Ser-Gly-Ser-(L-methioninase)-C. The peptide between amino acids 1–49 of urokinase A chain (designated ATF) and L-methioninase is a flexible linker designed to join the two proteins without disturbing their function and is not susceptible to cleavage by host proteases [11]. The ATF gene was amplified by PCR from the plasmid pULB1221 with a EcoRI restriction site added at the 5′ end and the flexible linker and a HindIII site added at the 3′ end. The L-methioninase gene contained in pKK223-3 was amplified by PCR with a BamHI site added at the 5′ end and a HindIII site at the 3′ end. PCR was performed using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, Ind.). After digestion with the appropriate restriction enzymes, PCR gene fragments were agarose gel-purified prior to ligation according to the Geneclean protocol (BIO101, Vista, Calif.). The digested and purified PCR fragments were directionally ligated into expression vector pKK223-3, which had been digested with EcoRI and HindIII and then purified by the Geneclean procedure. *E. coli* JM105 cells were transformed with the recombinant plasmid by electroporation.

Expression and purification of ATF-methioninase

A clone harboring the recombinant plasmid was grown to mid-log phase ($OD_{600\ nm} = 0.5$) at 37°C in shake flasks in 250 ml Luria broth medium containing 100 μg ampicillin and 1% glucose. At this point, the culture was induced with isopropyl-β-D-thiogalactoside at 1 m*M*, and an additional 100 μg ampicillin was added. The cells were grown for an additional 5 h and then harvested by centrifugation. The pellet was resuspended in 10 ml purification buffer at pH 8.0 (0.05 m*M* TPCK, 1 m*M* PMSF, 1% ethanol, 1 m*M* EDTA, 0.02 m*M* pyridoxal phosphate, 0.01% β-mercaptoethanol, 0.02 *M* Tris, pH 8.0). The suspended cells were sonicated at 4°C for a total of 2.5 min at 4.5 W/ml (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, Pa.). The lysate obtained was centrifuged at 12,000 *g* for 30 min to remove the cell debris and then was subjected to a heat treatment by holding at 50°C for 8 min and then cooling to 4°C. Subsequent steps were carried out at 4°C.

The lysate was fed onto a 40-ml column (2.5 cm diameter) of Q Sepharose Fast Flow anion exchange adsorbent (Amersham Biotech, Piscataway, N.J.) equilibrated with the purification buffer at pH 8.0, and the column was eluted with a linear gradient of 0–0.8 *M* KCl in purification buffer over 2 h at a superficial velocity of 30 cm/h. Ammonium sulfate was added to give 35% saturation to the pool of the fractions containing the fusion protein, and the precipitate was removed by centrifugation at 10,000 *g*. The supernatant was fed onto a 30-ml column (2.5 cm diameter) of Phenyl Sepharose 6 Fast Flow (Amersham Biotech) equilibrated with purification buffer at pH 6.5 and 35% saturated with ammonium sulfate. After washing the column with the same buffer that was 35% saturated with ammonium sulfate, the column was eluted with the same buffer with no ammonium sulfate. Both washing and elution for the hydrophobic interaction chromatography were at a superficial velocity of 30 cm/h. The fractions containing the fusion protein were dialyzed against purification buffer at pH 6.5 (0.05 m*M* TPCK, 1 m*M* PMSF, 1% ethanol, 1 m*M* EDTA, 0.02 m*M* pyridoxal phosphate, 0.01% β-mercaptoethanol, 0.02 *M* BisTris, pH 6.5). The dialyzed solution at pH 6.5 was fed onto the same anion exchange column as before, but with the column equilibrated with purification buffer at pH 6.5. The column was eluted under the same conditions as for the anion exchange chromatography at pH 8.0, except a 0–0.4 *M* KCl linear gradient was used. Fractions containing the fusion protein were pooled.

The enzymatic activity of L-methioninase was measured using L-methionine as a substrate and spectrophotometrically following the production of α-ketobutyrate with 3-methyl-2-benzothiazolone hydrazone hydrochloride [12]. Total protein was determined using the Bradford assay in a kit with bovine serum albumin as a standard (Bio-Rad, Richmond, Calif.). The Bradford protein assay was chosen because it gave much better protein balances around purification steps than the bicinchoninic acid (BCA) protein assay. Samples were analyzed by denaturing gel electrophoresis using the SDS-PAGE method with staining by Coomassie blue [13]. SigmaGel software (SPSS Science, Chicago, Ill.) was used to read band densities of Coomassie-stained gels. Amino-terminal protein sequencing was performed by the Molecular Biology Resource Facility at the University of Oklahoma, Health Sciences Center, on a protein sequencer equipped with an on-line PTH-amino acid analyzer (Procise model 492 sequencer, with model 610A data system; Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.).

Test compounds

Purified human urokinase was provided by Abbott Laboratories (Abbott Park, Ill.). Test compounds were dissolved in RPMI medium and control cultures received the same amount of RPMI medium alone.

Cell culture

MCF-7 human breast cancer cells were provided by the Michigan Cancer Foundation. The cells were maintained as monolayer cultures in RPMI 1640 medium (without phenol red) supplemented with 2 m*M* L-glutamine, gentamicin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), estradiol (10^{-11} *M*) (all from Sigma, St. Louis, Mo.) and 5% bovine calf serum (Hyclone, Logan, Utah) as previously described [14]. In some experiments, the culture medium was methionine-deficient or the methionine was replaced with homocystine. The medium was filter-sterilized and stored at 4°C prior to use. We have found that the MCF-7 cells used in this study are metastatic in a mouse xenograft model (unpublished observation).

Cell binding assay

The relative binding of the fusion protein to urokinase receptors was carried out by measuring the displacement of fusion protein. In the present study fusion protein displacement was quantified by measuring L-methioninase activity of the supernatant solution in response to increasing concentrations of pure urokinase. MCF-7 cells were plated in 96-well plates containing 10^3 cells/well, a saturating concentration of fusion protein (10^{-6} *M*), and pure urokinase over the range 3×10^{-10} to 3×10^{-6} *M*. Following a 1-h incubation at 37°C in an incubator containing a CO_2-enriched atmosphere, the supernatant was removed and centrifuged to remove all cellular debris. The supernatant L-methioninase concentration was measured using a spectrophotometric method as previously described [12]. The urokinase concentration that produced a 50% displacement of fusion protein in this assay was used to estimate the relative binding affinity as previously described [14].

Culture wounding assay

Cell migration and proliferation index were evaluated using the culture wounding assay. In previous studies we have used this method to quantify growth factor-mediated stimulation of MCF-7 cell migration [15]. By 3 days after seeding 5×10^5 MCF-7 cells into 60-mm culture dishes, the cells were approximately 90% confluent. The cultures were wounded, washed three times with phosphate-buffered saline, and treated with medium containing various concentrations of the fusion protein, methionine, or homocystine. In each experiment control cultures received RPMI medium alone. At 24 and 48 h, and in some cases 72 and 96 h, following treatment, cell migration and proliferation index were determined by measuring both the distance traveled by the cell front into the wounded area (migration) and the number of cells in the wounded area (proliferation index) per microscopic field. Measurements were taken from 10 to 12 individual microscopic fields in each experiment, and the data from two or three experiments are summarized.

Data analysis

Multiple group comparisons were conducted using ANOVA and Student's *t*-test for pair-wise comparisons. Group differences resulting in *P* values of less than 0.05 were considered to be statistically significant.

Results

Expression and purification of ATF-methioninase

The ATF-methioninase fusion protein was expressed in soluble form in *E. coli* JM105 cells after transformation with the recombinant plasmid. The SDS-PAGE results shown in Fig. 1 show the overexpression of the ATF-methioninase fusion protein in a clone containing the recombinant plasmid and the increasing purity of the fusion protein as the purification progressed. The purity of the fusion protein in the pooled fractions from the final chromatography was estimated to be 98% using SigmaGel densitometry software, and the specific L-methioninase activity for these pooled fractions was 3.6 U/mg total protein (18 times higher than the specific L-methioninase activity in the starting cell lysate). The measured recovery of L-methioninase activity during purification was 29%.

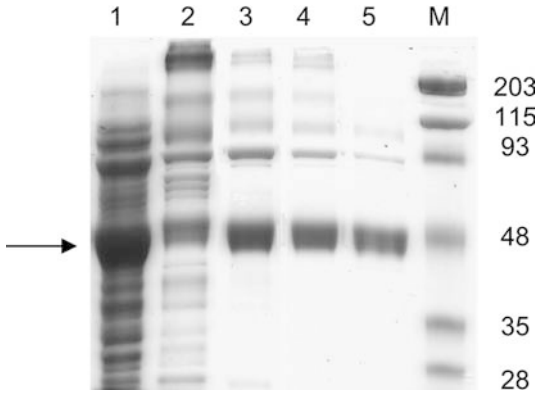

Fig. 1 SDS-PAGE analysis with Coomassie blue staining of the expression and purification of ATF-methioninase fusion protein (position indicated by the *arrow*). The fusion protein was expressed from plasmid pKK223-3 under the control of the *tac* promoter in *E. coli* JM105 at 37°C (*lane 1* soluble lysate, *lane 2* heat-treated soluble lysate, *lane 3* pooled fractions from anion exchange chromatography at pH 8.0, *lane 4* pooled fractions from hydrophobic interaction chromatography, *lane 5* pooled fractions from anion exchange chromatography at pH 6.5; *M* molecular weight markers indicated on the right in kiloDaltons)

Sequencing of the purified fusion protein was performed on the first eight amino-terminal amino acids. The sequence was identical to the amino-terminus of the urokinase A chain (Ser-Asn-Glu-Leu-His-Gln-Val-Pro) for $80\pm5\%$ of the protein. One of the minor sequences obtained, for 10% of the protein, was the same as the sequence of the urokinase A chain starting at amino acid 21, which indicates that the first 20 amino acids were cleaved off (between Val and Ser) to give this sequence. This apparent cleavage of the fusion protein may explain the width of the band corresponding to the fusion protein in the SDS-PAGE analysis (Fig. 1). Thus, the sequences for the fusion protein, either whole or with the first 20 amino acids cleaved off, account for $90\pm5\%$ of the protein, which is close to the purity determined by SDS-PAGE when error in the sequencing results is taken into account.

Methionine dependency of MCF-7 cells

In order to determine the methionine dependency of the MCF-7 cells used in this study, the growth of cells in

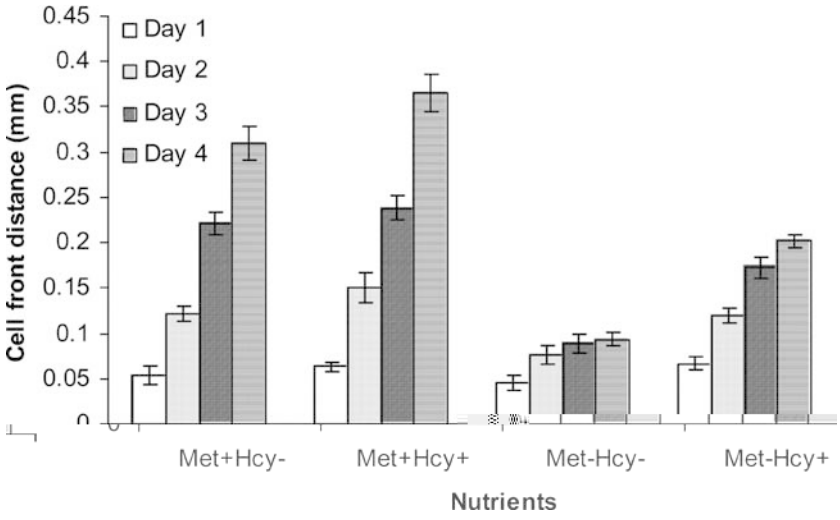

Fig. 2 Effects of methionine deficiency on MCF-7 cell migration. *Each bar* represents the distance (mean ± SEM from 10 to 12 microscopic fields) of cell migration into the wounded area (*Met+* methionine concentration 15 mg/l, *Hcy+* homocystine concentration 15 mg/l, *Met-* absence of methionine, *Hcy-* absence of homocystine in the medium)

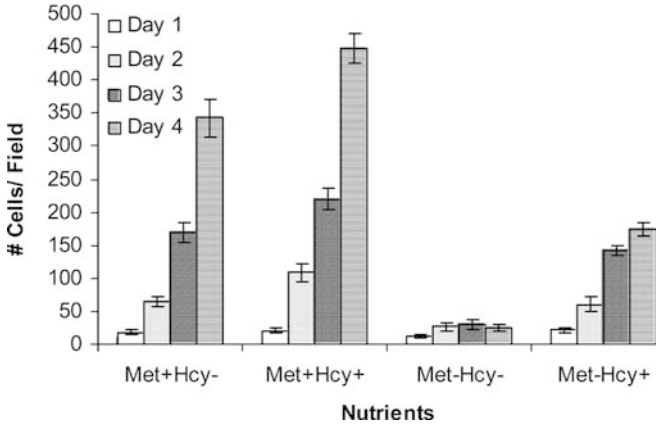

Fig. 3 Effects of methionine deficiency on MCF-7 cell proliferation index. *Each bar* represents the number of cells in the wounded area (mean ± SEM from 10 to 12 microscopic fields) (*Met+* methionine concentration 15 mg/l, *Hcy+* homocystine concentration 15 mg/l, *Met-* absence of methionine, *Hcy-* absence of homocystine in the medium)

complete medium and methionine-free medium were compared. Cell migration and the proliferation index were significantly reduced in the absence of methionine on days 1–4 ($P < 0.05$; Figs. 2 and 3). In addition, the addition of homocystine improved cell migration and proliferation, but did not reverse the effects of methionine deficiency.

Inhibitory effects of ATF-methioninase

The effects of fusion protein were examined over the concentration range 10^{-6} to 10^{-8} *M* as shown in Figs. 4 and 5. In these experiments, the fusion protein produced a dose-related inhibition of both the migration and proliferation index of MCF-7 cells on days 2 and 3 following fusion protein treatment ($P < 0.05$). On day 1, the fusion protein-induced inhibition of cell migration and the proliferation index was found not to be dose-related.

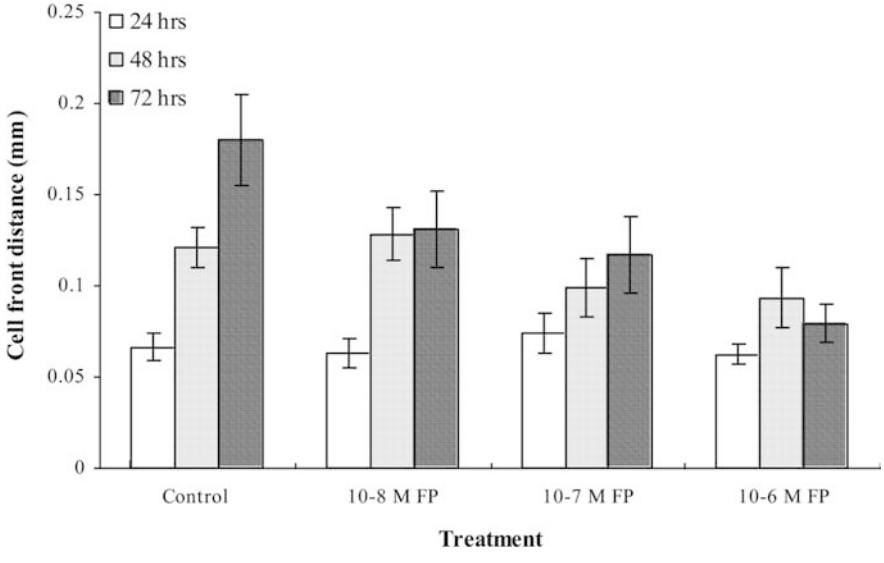

Fig. 4 Dose-response effect of the fusion protein on MCF-7 cell migration. *Each bar* represents the distance (mean ± SEM from 10 to 12 microscopic fields) of cell migration into the wounded area

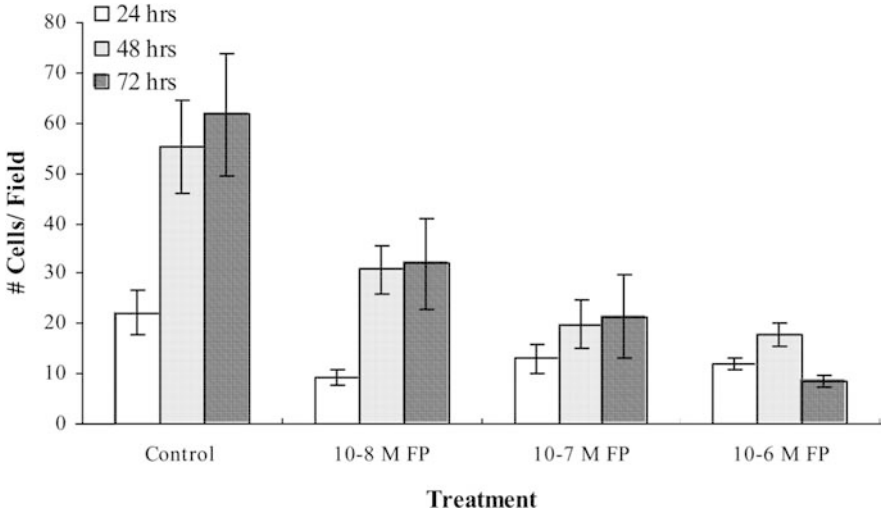

Fig. 5 Dose-response effect of fusion protein on MCF-7 cell proliferation index. *Each bar* represents the number of cells in the wounded area (mean ± SEM from 10 to 12 microscopic fields)

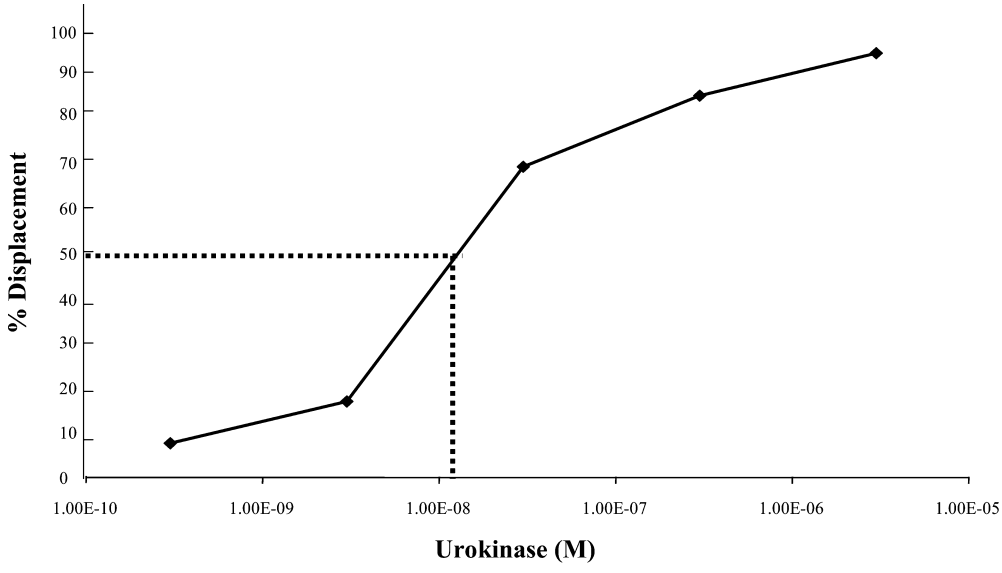

Fig. 6 Urokinase-induced displacement of fusion protein from membrane binding sites in MCF-7 cells. The data presented are summarized from two experiments. The *dotted line* indicates the concentration of human urokinase that produced a 50% displacement of fusion protein

Specific binding of ATF-methioninase to MCF-7 cells

In these experiments, the displacement of the fusion protein from urokinase receptors on MCF-7 cells was determined. As shown in Fig. 6, increasing concentrations of urokinase over the concentration range 3×10^{-10} to 3×10^{-6} *M* produced a dose-related displacement of fusion protein. The urokinase concentration necessary to produce a 50% displacement of fusion protein was determined to be approximately 10^{-8} *M*. It was also determined that human EGF, over the same concentration range, did not produce any significant displacement of the fusion protein.

Discussion

We constructed the fusion protein ATF-methioninase by ligating the gene for the first 49 amino acids of the urokinase A chain to a gene for L-methioninase from *P. putida*, with the gene coding for a six amino acid flexible linker in between. This fusion protein, which had L-methioninase activity, was produced in *E. coli* in soluble form and purified to near homogeneity with three chromatography steps.

Amino acids 1–49 of the urokinase A chain (denoted ATF) were used in the fusion protein since this includes residues 12–32 that have been shown to be critical for binding to the urokinase receptor [8]. The kringle domain of the urokinase A chain was excluded because this domain has been shown to bind heparin, which could bind polyanionic molecules such as the proteoglycans and aid in the invasion of tissue [16]. Since the fusion proteins were produced in recombinant *E. coli*, the threonine at residue 18 of the urokinase fragment would not be fucosylated; thus, the urokinase fragment would not have the undesirable cell-proliferation property of the corresponding human urokinase fragment, which is

dependent on Thr^{18} being fucosylated [17]. Bacteria such as *E. coli* do not carry out post-translational glycosylations such as fucosylation.

The MCF-7 breast cancer cells used in the biological testing were verified as methionine-dependent, as demonstrated by the reduction in cell migration and proliferation index when the amino acid methionine was replaced by homocystine (Figs. 2 and 3). Normal human cell lines survive and grow well with this substitution. The ATF-methioninase fusion protein in *E. coli* cell lysate inhibited the migration and proliferation index of MCF-7 cells over the concentration range 10^{-6} to 10^{-8} *M* in a dose-dependent manner over a period of 3 days (Figs. 4 and 5). To show that ATF-methioninase would bind specifically to MCF-7 cells, a binding assay was performed by saturating the cells with the fusion protein and adding urokinase at various concentrations. The relative affinity of ATF-methioninase for the cells (50% competition at 10^{-8} *M*, Fig. 6) is 10-fold lower than that reported by others for the displacement of a larger ATF (1–135) by urokinase from cultured cells [7], which consistent with reduced binding strength for other urokinase ATF's that are less than 135 amino acids in size [8].

The major mechanism of the inhibitory effects of ATF-methioninase in this study is believed to be the methioninase-induced depletion of methionine available to the cells. Another possible mechanism of ATF-methioninase inhibition of cell migration and proliferation may be related to the specific binding to, and inactivation of, the urokinase receptor. Since urokinase is known to be involved in cancer cell invasion, specific binding to this receptor, by fusion protein, may inhibit or alter urokinase-related activity.

Urokinase or ATF have been fused to the cytotoxic proteins saporin [18] and diphtheria toxin [19]. While these fusion proteins were found to be cytotoxic to cancer cells, they would also kill normal cells that also have urokinase receptors, such as neutrophils, eosinophils, monocytes, and fibroblasts. The ATF-methioninase fusion protein is advantageous in this respect, since the growth of normal cells would not be inhibited.

One possible drawback to the use of L-methioninase in human cancer therapy is the immunogenicity of bacterially derived L-methioninase. Since L-methioninase has shown no toxicity in mice in previous studies [4], immunogenicity may not be a significant concern. However, one way to reduce a protein's immunogenicity is to conjugate it to polyethylene glycol (PEG) [20]. L-Methioninase has, in fact, been successfully conjugated to PEG, resulting in a significant increase in serum half-life in rats [21].

None of the work to date with L-methioninase treatment of cancer cells has involved the targeting of the L-methioninase to the cell surface. Advantages that are foreseen for targeting L-methioninase to the cell surface include the following. Since the L-methioninase is being targeted to the cells that it is intended to kill, the dosages of this fusion protein containing L-methioninase should be much lower than when L-methioninase alone is administered systemically. In addition, it may be possible to avoid having dietary restrictions of methionine, homocystine, and choline and injections of homocystine as were needed in the previous studies with mice [4].

In conclusion, the results of this study demonstrate that an ATF-methioninase fusion protein is capable of inhibiting both the proliferation and migration of human breast cancer cells. In addition, our results indicate that the fusion protein is specifically targeted to the urokinase receptor of the cancer cells. This fusion protein may serve as a prototype for targeting methioninase and/or other cytotoxic agents to metastatic cancer cells.

Acknowledgements The authors acknowledge the support of the University of Oklahoma, Bioengineering Center. This work was also supported in part by grants from NIH/NCI (CA-89740), DoD (DAMD17-01-1-0591) and OCAST (HR03-046).

References

1. Halpern BC, Clark BR, Hardy DN, Halpern RM, Smith RA (1974) The effect of replacement of methionine by homocystine on survival of malignant and normal adult mammalian cells in culture. Proc Natl Acad Sci U S A 71:1133
2. Mecham JC, Rowitch D, Wallace CD, Stern PH, Hoffman RM (1983) The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys Res Commun 117:429
3. Kokkinakis DM, von Wronski MA, Vuong TH, Brent TP, Schold SC (1997) Regulation of O^6-methylguanine-DNA methyltransferase by methionine in human tumour cells. Br J Cancer 75:779
4. Kokkinakis DM, Schold SC, Hori H, Nobori T (1997) Effect of long-term depletion of plasma methionine on the growth and survival of human brain tumor xenografts in athymic mice. Nutr Cancer 29:195
5. Tan Y, Zavala J, Xu M, Zavala J Jr, Hoffman RM (1996) Serum methionine depletion without side effects by methioninase in metastatic breast cancer patients. Anticancer Res 16:3937
6. Ellis V, Pyke C, Eriksen J, Solberg H, Dano K (1992) The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. Ann N Y Acad Sci 667:13
7. Stoppelli MP, Corti A, Soffientini A, Cassani G, Blasi F, Assoian RK (1985) Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci U S A 82:4939
8. Appella E, Robinson EA, Ullrich SJ, Stoppelli MP, Corti A, Cassani G, Blasi F (1987) The receptor-binding sequence of urokinase. J Biol Chem 262:4437
9. Hori H, Takabayashi K, Orvis L, Carson DA, Nobori T (1996) Gene cloning and characterization of *Pseudomonas putida* L-methionine-α-deamino-γ-mercaptomethane-lyase. Cancer Res 56:2116
10. Jacobs P, Cravador A, Loriau R, Brockly F, Colau B, Chuchana P, Van Elsen A, Herzog A, Bollen A (1985) Molecular cloning, sequencing, and expression in *Escherichia coli* of human preprourokinase cDNA. DNA 4:139
11. Argos P (1990) An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. J Mol Biol 211:943
12. Esaki N, Soda K (1973) L-methionine γ-lyase from *Pseudomonas putida* and *Aeromonas*. Methods Enzymol 143:459
13. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680

14. Jain PT, Rajah TT, Pento JT (1997) Antitumor activity of a novel antiestrogen (Analog II) on human breast cancer cells. Anticancer Drugs 8:964
15. Nguyen TT, Zang XP, Pento JT (2002) Effects of KGF inhibitors on the migration and proliferation of breast cancer cells in a culture wounding model. Pharmacol Res 46:79
16. Stephens RW, Bokman AM, Myohanen HT, Reisberg T, Tapiovaara H, Pedersen N, Grondahl-Hansen J, Llinas M, Vaheri A (1992) Heparin binding to the urokinase kringle domain. Biochemistry 31:7572
17. Rabbani SA, Mazar AP, Bernier SM, Haq M, Bolivar I (1992) Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. J Biol Chem 267:14141
18. Cavallaro U, del Vecchio A, Lappi DA, Soria MR (1993) A conjugate between human urokinase and saporin, a type-1 ribosome-inactivating protein, is selectively cytotoxic to urokinase receptor-expressing cells. J Biol Chem 268:23186
19. Vallera DA, Li C, Jin N, Panoskaltsis-Mortari A, Hall WA (2002) Targeting urokinase-type plasminogen activator receptor on human glioblastoma tumors with diphtheria toxin fusion protein DTAT. J Natl Cancer Inst 17:597
20. Abuchowski A, van Es T, Palczuk NC, Davis FF (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem 252:3578
21. Tan Y, Sun X, Xu M, An Z, Tan X, Han Q, Miljkovic DA, Yang M, Hoffman RM (1998) Polyethylene glycol conjugation of recombinant methioninase for cancer therapy. Protein Expr Purif 12:45