Influence of L-Methioninase Targeted to the Urokinase Receptor on the Proliferation and Motility of Lung and Prostate Cancer Cells

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Abstract. *Background: Previously, we reported that a novel fusion protein consisting of an amino-terminal fragment of urokinase linked to the amino terminus of the enzyme L-methioninase inhibited MCF-7 breast cancer cells in vitro to a greater extent than treatment with L-methioninase. Materials and Methods: The fusion protein, L-methioninase and a mutated fusion protein without L-methioninase activity were produced by recombinant methods. The effects of fusion protein, L-methioninase, and mutated fusion protein treatment on the proliferation and motility of SK-LU-1 lung and PC-3 prostate and cancer cells were measured in vitro using a culture wounding assay. Results: The fusion protein produced a dose-dependent inhibition of the proliferation and motility of both cancer cell lines. In addition, the fusion protein was found to be significantly more effective than L-methioninase alone or mutated fusion protein. Conclusion: Our results suggest that this fusion protein has potential as a selective therapeutic agent for the treatment of various methionine-dependent cancers.*

Methionine dependence is a metabolic defect that has been observed in numerous cancer cell lines (1, 2). These methioninase-dependent cancer cells are unable to proliferate or survive when the amino acid methionine (Met) is replaced in the medium with homocystine (Hcy). However, normal adult cell lines survive and grow well with this substitution. The methionine-dependence of cancer cells has led to dietary and pharmacological research designed to exploit this property for the treatment of cancer (3). However, animals are not able to tolerate a long-term Met- and Hcy-deficient diet. In one study in which mice with human cancer xenografts were fed a Met-deficient diet, it was observed that tumor growth was greatly inhibited, while the animal body weight was maintained by the once-weekly administration of methionine (4). Another approach has been to administer the enzyme L-methioninase which degrades methionine. The *i.v.* infusion of L-methioninase for 24 hours to nine patients with either advanced lung, breast or kidney cancer, or lymphoma in a phase I clinical trial resulted in the reduction of serum methionine to very low levels, with no adverse side-effects (5).

With the goal of developing a more effective L-methioninase cancer treatment with greater cancer cell selectivity and cytoxicity, utilizing less enzyme and shorter treatment periods, we designed a fusion protein consisting of an amino-terminal fragment of human urokinase (ATF, amino acids 1-49) linked to the amino-terminal of L-methioninase (6). The L-methioninase is targeted to the urokinase receptor because the urokinase receptor is over-expressed by many cancer cells (7-13) and because both urokinase and its receptor are found consistently at the invasive foci of most types of human cancer (14). The ability of cancer cells to degrade extracellular matrices is critical to the process of cancer metastasis and urokinase is responsible for the generation of plasmin which is involved in the process of extracellular matrix degradation. Others have demonstrated that the A chain of urokinase is responsible for binding to the receptor (15) and that residues 12-32 in the A chain are critical for receptor binding (16). Furthermore, it has been shown that the amino-terminal fragment of the A chain, consisting of residues 1-135 is not internalized, since the catalytic domain in the B chain is required for internalization (17).

Abbreviations: ATF, amino-terminal fragment of urokinase (amino acids 1-49); PMSF, phenylmethylsulfonyl fluoride.

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Our previous studies with ATF-methioninase in breast cancer demonstrated that this fusion protein binds specifically to the urokinase receptor of MCF-7 cells *in vitro*, based on the measurement of ATF-methioninase displacement by urokinase (6) and by immunocytochemical localization (18). Moreover, ATF-methioninase produced a dose dependent inhibition of both the proliferation and migration of MCF-7 cells *in vitro* and a reduction in the growth of MCF-7 tumor xenografts *in vitro* (18).

Because of our earlier promising results with ATF-methioninase in breast cancer cells, the present study was designed to examine the influence of this fusion protein on lung and prostate cancer cells, which are known to be methionine dependent. Further, this study compared the activity of the fusion protein to free L-methioninase and a fusion protein containing a mutated (and inactive) methioninase. The L-methioninase and mutated fusion protein were included as controls to demonstrate the targeting specificity and importance of methioninase activity for the inhibition of cancer cell growth and migration.

Materials and Methods

Materials. Synthetic oligonucleotides were produced by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center. Vector pET-30 EK/LIC, HRV 3C protease, and NovaBlue and BL21(DE3) *Escherichia coli* cells were obtained from Novagen (Madison, WI, USA).

Construction, expression and purification of recombinant proteins. E. coli host strain BL21(DE3), harboring the genes for ATF-methioninase, mutated ATF-methioninase (Y114F mutation), or L-methioninase, was employed for the expression of the three recombinant proteins used in this study. The construction, expression and purification of the proteins used in this study were accomplished as described elsewhere (18). DNA sequences were verified by sequencing at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA). Amino-terminal protein sequencing was performed by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center.

Cell culture. SK-LU-1 and PC-3 human cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained as monolayer cultures in RPMI 1640 media (without phenol red) supplemented with 2 mM L-glutamine, gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), estradiol (10^{-11} M) (all from Sigma, St. Louis, MO, USA), and 5% bovine calf serum (Hyclone, Logan, UT, USA) as described elsewhere (19). The media was filter sterilized and stored at 4˚C prior to use.

Culture wounding assay. Cell migration and proliferation were evaluated using the culture wounding assay as described elsewhere (6, 19). Three days after seeding $5x10^5$ cancer cells into 60-mm culture dishes, when the cells were approximately 80% confluent, the cultures were wounded, washed three times with phosphate-buffered saline (PBS) and treated with media containing either fusion protein, mutated fusion protein, or L-methioninase. In each experiment, control cultures received RPMI media alone. At 24, 48 and 72 hours following treatment, cell migration and proliferation 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) per microscopic field. Measurements were taken from ten to twelve individual microscopic fields in each experiment and data were summarized from two to three experiments.

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

Inhibitory effects of fusion protein, mutated fusion protein and L-methioninase on SK-LU-1 lung cancer cells. The effects of ATF-methioninase fusion protein on SK-LU-1 lung cancer cells were examined over a concentration range of 10^{-6} to 10^{-8} M as shown in Figures 1 and 2. In these experiments, the fusion protein consistently produced a statistically significant dose-related inhibition of both proliferation (Figure 1) and migration (Figure 2) of the lung cancer cells on days 2 and 3 following fusion protein treatment at all concentrations compared to the control. The fusion protein with a mutated L-methioninase produced little or no inhibition of cell proliferation or migration over the same concentration range. Treatment of SK-LU-1 cancer cells with L-methioninase alone produced a significant inhibition of cell migration on days 2 and 3 following treatment only at the highest concentration of 10^{-6} M; proliferation was significantly inhibited on day 2 only at 10^{-6} M but was not inhibited on day 3 at any of the concentrations. Thus, L-methioninase-induced inhibition was significantly ($p<0.05$) less effective at each treatment day than that produced by the fusion protein.

Inhibitory effects of fusion protein, mutated fusion protein and L-methioninase on PC-3 prostate cancer cells. The effects of ATF-methioninase fusion protein on PC-3 prostate cancer cells were examined over a concentration range of 10^{-6} to 10^{-8} M as shown in Figures 3 and 4. In these experiments, the fusion protein consistently produced a statistically significant dose-related inhibition of both the proliferation and migration of the cancer cells on days 2 and 3 following fusion protein treatment at all concentrations. The fusion protein with a mutated L-methioninase again produced little or no effect on cell proliferation or migration over the same concentration range. Treatment of the PC-3 cancer cells with L-methioninase alone consistently produced a significant reduction in proliferation and migration on all three days only at the highest concentration of 10^{-6} M. The L-methioninase-induced inhibition was significantly less than that produced by the fusion protein on day 3 at all concentrations tested ($p<0.05$).

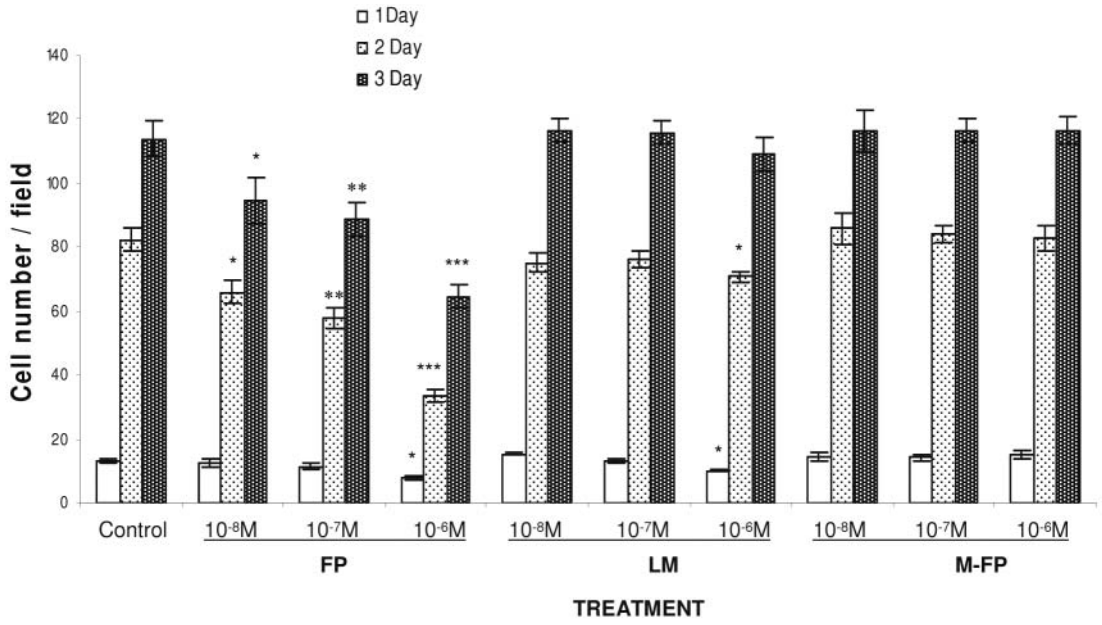

Figure 1. *Dose-response effect of fusion protein on SK-LU-1 lung cancer cell proliferation. Fusion protein (FP), mutated FP (M-FP), or L-methioninase (LM) were administered immediately following culture wounding. Each bar represents the number of cells that migrated into the wounded area (mean±SEM from 10 to 12 microscope fields). Significantly different from the Control; *p<0.05, **p<0.01, ***p<0.005.*

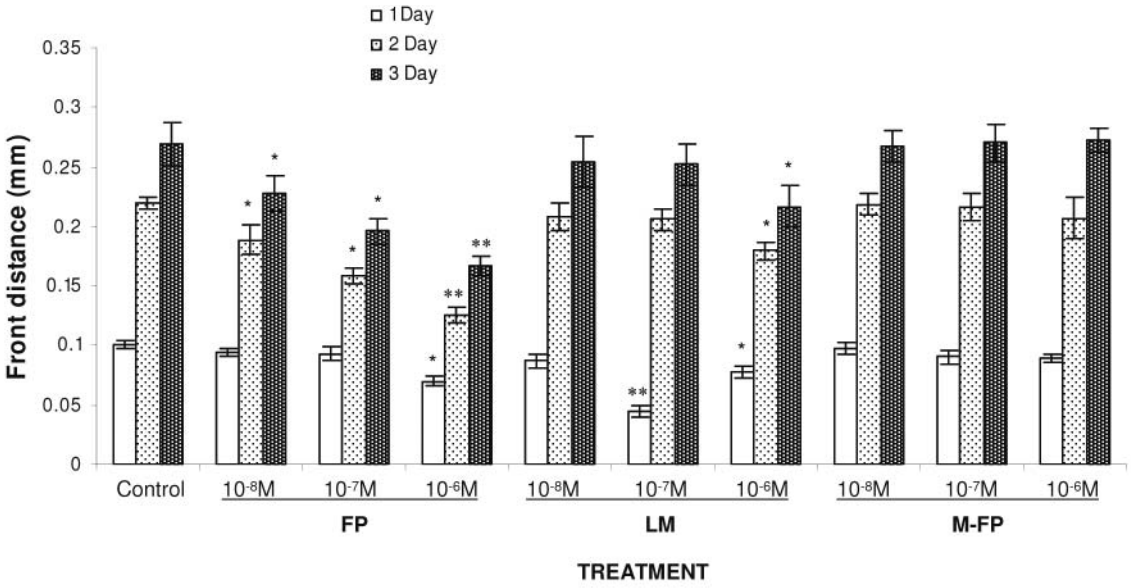

Figure 2. *Dose-response effect of fusion protein on SK-LU-1 lung cancer cell migration. Fusion protein (FP), mutated fusion protein (M-FP), or L-methioninase (LM) were administered immediately following culture wounding. Each bar represents the distance of cell migration into the wounded area (mean±SEM from 10 to 12 microscope fields). Significantly different from the Control; *p<0.05, **p<0.01.*

Discussion

We have previously reported that the ATF-methioninase fusion protein inhibited proliferation and migration of MCF-7 breast cancer cells both in cell culture and in nude mouse xenografts *in vivo* (6, 18). The present study compared the effects of this same ATF-methioninase fusion protein to a methioninase-mutated fusion protein and L-methioninase alone on SK-LU-1 lung cancer and PC-3 prostate cancer cell lines. The SK-LU-1 and PC-3 cancer

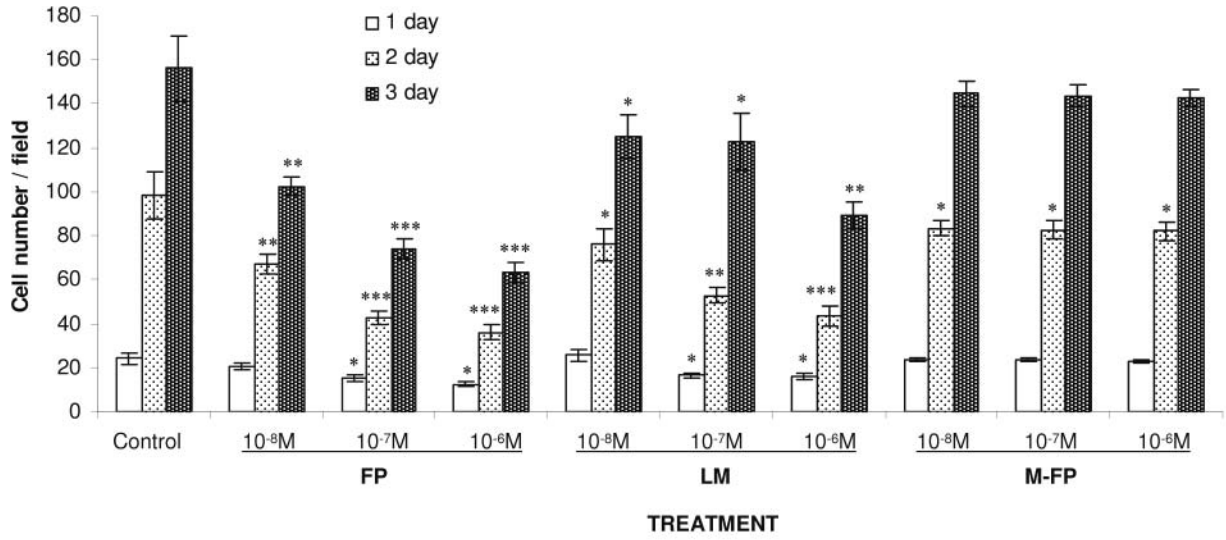

Figure 3. *Dose-response effect of fusion protein on PC-3 prostate cancer cell proliferation. Fusion protein (FP), mutated FP (M-FP), or L-methioninase (LM) were administered immediately following culture wounding. Each bar represents the number of cells that migrated into the wounded area (mean±SEM from 10 to 12 microscope fields). Significantly different from the Control; *p<0.05, **p<0.01, ***p<0.005.*

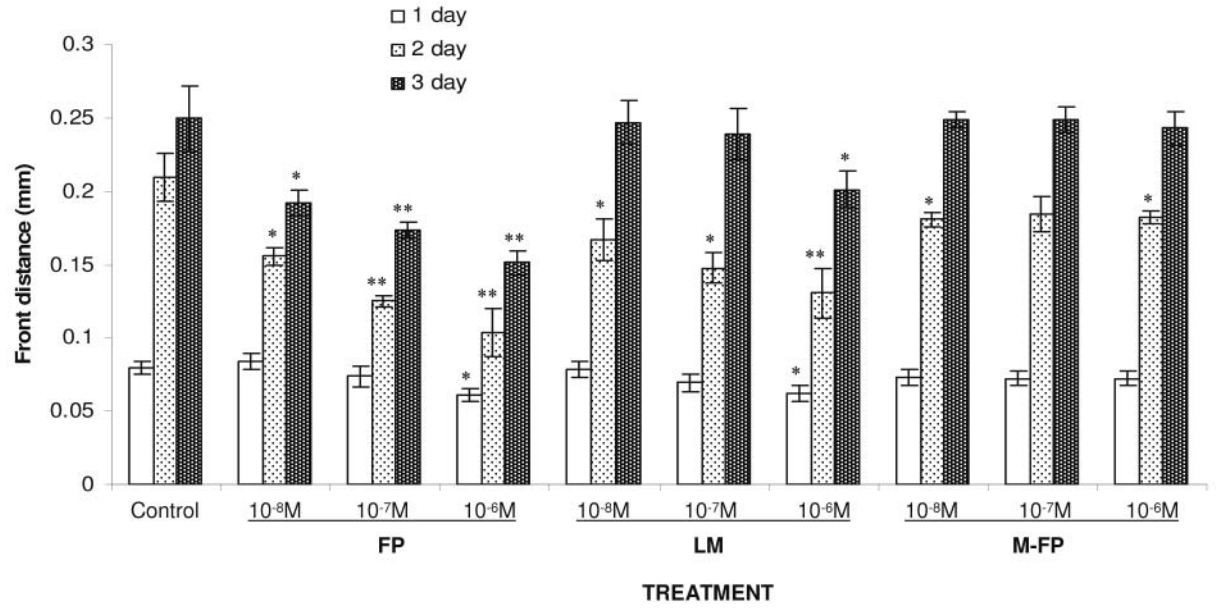

Figure 4. *Dose-response effect of fusion protein on PC-3 prostate cancer cell migration. Fusion protein (FP), mutated fusion protein (M-FP), or L-methioninase (LM) were administered immediately following culture wounding. Each bar represents the distance of cell migration into the wounded area (mean±SEM from 10 to 12 microscope fields). Significantly different from the Control; *p<0.05, **p<0.01.*

cell lines were chosen for study because they have been shown to be methionine dependent (20) and to express the urokinase receptor (7, 8).

The ATF-methioninase fusion protein inhibited the proliferation and migration of both cancer cell lines over a concentration range of 10^{-6} to 10^{-8} M in a dose-dependent manner over a period of 3 days (Figures 1-4). The fusion protein was observed to be significantly more effective than free L-methioninase in inhibiting cancer cell proliferation and migration, which supports our rationale for targeting L-methioninase to the surface of the cancer cells. In addition, the lack of inhibition with the mutated fusion protein in this study demonstrates that it is the L-methioninase activity at the cell surface and not competitive inhibition at the urokinase receptor site that is responsible for the anticancer effects observed.

It is clear that the major mechanism of the inhibitory effects of ATF-methioninase in this study is the methioninase-induced depletion of methionine available to the cancer cells. The use of ATF to target L-methioninase to urokinase receptors on the surface of cancer cells is advantageous for membrane targeting because the ATF fragment of urokinase is not internalized. Internalization of the ATF protein does not occur since the catalytic domain of urokinase, which is required for internalization, is not present in the ATF protein (17). There are many receptors on these lung and prostate cancer cells, and most of these receptors are internalized following ligand binding and thus would not be useful for targeting L-methioninase to the cell surface.

In conclusion, the ATF-methioninase fusion protein was found to be significantly more effective than free L-methioninase as an anticancer agent. Thus, the ATF-mediated targeting of methioninase activity to the surface of the cancer cells appears to be an effective approach and may serve as a therapeutic prototype for the development of a new class of highly effective, targeted chemotherapeutic agents.

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