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13. ABSTRACT (Maximum 200 Words)

The object of this proposal is to understand the tumor suppressor function of maspin in prostate, and to explore maspin's role in normal prostate development. Maspin knockout mouse model will be employed to study the effects of loss of maspin function on mouse prostate tumorigenesis and development. We hypothesize that the presence of maspin (by stable transfection or by adenovirus mediated maspin gene delivery) will prevent or delay prostatic tumorigenesis and metastasis, while loss of maspin in mouse model will render it more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing maspin knockout mice with a well characterized mouse prostate cancer model (TRAMP) to test this hypothesis. Prostatic tumorigenesis and normal prostate development will be studied using a variety of established techniques, including organ culture, histopathology, and molecular biology.

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Ming Zhang, Ph.D. 2/12/04

PI - Signature Date

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Introduction

Prostate cancer is the most commonly diagnosed cancer in U.S. males. Due to the severity of this malignant disease, research of prostate needs to be focused on the treatment of prostate cancer and scientists in this field are obligated to accelerate the process of translation of their basic research into clinical usage. The object of this proposal is to understand the tumor suppressor function of maspin in prostate, and to explore maspin's role in normal prostate development. Maspin knockout mouse model will be employed to study the effects of loss of maspin function on mouse prostate tumorigenesis and development. We hypothesize that the presence of maspin (by stable transfection or by adenovirus mediated maspin gene delivery) will prevent or delay prostatic tumorigenesis and metastasis, while loss of maspin in mouse model will render it more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing maspin knockout mice with a well-characterized mouse prostate cancer model (TRAMP) to test this hypothesis. Prostatic tumorigenesis and normal prostate development will be studied using a variety of established techniques, including organ culture, histopathology, and molecular biology.

The specific aims for this three-year proposal are:

Aim 1. Examination of maspin as a tumor suppressor in prostate. I plan to test whether maspin inhibits tumor growth in cell culture and tumor progression in athymic mice. We will deliver maspin to prostate tumors by adenovirus mediated gene delivery technique. In this way, the effectiveness of maspin as a therapeutic agent can be directly evaluated.

Aim 2. Examination of maspin knockout mice on tumor progression and normal prostate development. The effect of maspin gene disruption on prostatic tumorigenesis will be tested by crossing maspin KO mice with TRAMP mice. Maspin knockout mice will also be used to evaluate the loss of maspin on prostate development. The KO mice will be valuable to support our hypothesis if these mice are more susceptible to tumorigenesis and metastasis because of the loss of tumor suppressor, maspin.

Body

Materials and methods

Animals

Syngeneic C57BL/6 mice (for implantation of TRAMP tumor cells) were purchased from Harlan, Inc. All animals were maintained within the PI's animal facility at Baylor.
TRAMP cell lines were obtained from Dr. Norm Greenberg at Baylor College of Medicine. Maspin hetrozygous knockout mice were generated in PI's laboratory.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. All secondary antibodies were purchased from Zymed, Inc. Antibodies to E-cadherin, ZO-1, PCNA were from Zymed, Pharmingen Inc.

Northern and Western analysis

RNAs and proteins were isolated from cells, prostate tissues. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 ug RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Prostate tissues were removed from male mice and dissected. Tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin (AbS4A). The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For PCNA staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit.

Early prostate morphogenesis
Prostate tissues were dissected from day2-day 8 pups and the nodular structures were examined under microscope. The number of nodules was counted.

Histology of old prostate tissues
Mice of 6 to 12 months of age were biopsied and different prostate structures were dissected and processed for histology analysis.

Results and Discussion

Task 1. Examination of maspin as a tumor suppressor in prostate.

We have overexpressed maspin in TRAMP prostate tumor cells by retrovirus infection. Retroviral stable transfectants were constructed. The plasmid constructs pS2-maspin GFP and pS2-GFP were transfected into 293T cells, along with pECO plasmid using fugene reagent, to produce infective viral particles. The viral supernatants were then allowed to infect C2N TRAMP tumor cells in the presence of polybrene. The transduced cells were then selected in presence of 100 ug/ml of zeocin for four weeks. These cells were subsequently sorted by flow cytometry for green fluorescence emitted by GFP. The retroviral stable transfectants were further selected as individual clones from 96 well titer plates. These retroviral stable transfectants were then analyzed for the presence of human maspin cDNA by RT-PCR. The expression of maspin cDNA was detected by Western blotting by mouse monoclonal antibody to human maspin (Pharmigen) and by immunostaining with ABS4a antibody to mouse maspin.

These retroviral stable transfectants will be further characterized by *in vitro* experiments, by comparing the maspin transfectants versus the vector-alone transfectants and parental C2N cells, for their growth rate, anchorage-independent growth on soft agar, and tumorigenicity. The tumor cell invasion and metastasis will be compared *in vitro* by cell adhesion and motility assays. We will also analyze the changes in cell adhesion in the presence of various extracellular matrix (ECM) proteins and thereby investigate their ability to attach to the basement membrane. This study was published in the Journal of Urology in 2003; an editorial paper for our study was published in the same issue.

We have carried out in vivo experiment to examine the tumorigenic potential of these transfectants in syngenic mice. For this purpose, parental C2N cells and maspin stable cells are injected subcutaneously into C57BL/6 mice. Tumor growth rate in vivo will be monitored and samples are taken for analysis. Surprisingly, most of C2N cells inoculated developed into palpable tumors. However, none of the maspin stable clones have developed any tumors after extended time of observation, demonstrating those maspin functions to inhibit prostate tumor in vivo. To determine other factors which may contribute to maspin's tumor suppression property, we utilized a tumor spheroid 3D-culture system. We grew the control C2N cells and C2N-46 maspin expressing cells in 3D-tumor spheroids. These spheroids were harvested, embedded in paraffin, and sectioned to 5 μm slides for H&E histology. Control C2N and maspin-expressing C2N-46 tumor cells both formed 3D tumor spheroid, and H&E histology showed that tumor cell morphology was similar to that of the C2N tumors in mice. However, when we examined sections of tumor spheroids for apoptosis rate by the TUNEL assay, C2N-46 tumor cells had a significant higher rate of apoptosis than that of C2N control (see attached appendix). This data suggest maspin induced apoptosis may represent a major contributing factor for the inhibition of prostate tumor growth in vivo.

Another important factor for tumor growth in vivo is that tumor cells require vasculature network to support their growth. Since we have demonstrated that maspin is an angiogenesis inhibitor in other system, we determine whether C2N-46 maspin transfectants secret maspin to inhibit angiogenesis. We cultured C2N-46 and C2N cells and collected conditional medium from both cell cultures. Western blot analysis demonstrated that C2N-46 cells were capable of secreting maspin protein to the conditional medium .We then examined the ability of the C2N-46 condition medium to affect human umbical endothelial cell (HUVEC) migration by a modified Boyden chamber assay. HUVEC cells were subjected to the treatment of condition medium from C2N-46 cells or C2N control cells. Significant differences in cell migration were observed between control and C2N-46 medium treated samples ($p<0.003$). Maspin-blocking antibody was used in the migration assay. Addition of maspin antibody blocked maspin's inhibitory effect on migration, while control preserum had no adverse effect. These data indicate that C2N-46 transfectants expressed sufficient amount of maspin. These maspin proteins were effectively secreted and acted to inhibit the endothelial cell migration. This study has recently been submitted and the manuscript was attached in the appendix.

Task 2. The effect of maspin gene disruption on tumor progression and normal prostate development.

Because homozygous deletion of maspin is lethal, we decided to assess the partial loss of maspin on tumor progression. We hypothesize that maspin heterozygous mice may display gene dosage phenotype in prostate. Therefore, we crossed the maspin KO mouse with the TRAMP mouse for tumor study in late 2000. However, a tropical storm in the June of 2001 had wiped out most of the maspin knockout mice and the mice for this tumor study. Due to time limit, we designed an

alternative approach. This involves implanting maspin wildtype and knockout ES cells into kidney capsule of wildtype mice and compare the tumor growth rate of these KO ES cells with wildtype ES cells in vivo. Our result was not conclusive partly due to the lack of method to monitoring kidney tumor growth rate and relative small number of animals planned for this experiment. In addition, we fear that the kidney tumor may not be able to completely mimic what happens in prostate cancer. Facing the time limitation of this grant, we decided to focus rather on the second part of this specific aim. That is to study the deletion of maspin on normal prostate development.

The study of maspin deletion on prostate development has generated fruitful information. We specifically examined two different time courses of prostate development. One is the early prostate ductal morphogenesis and the other is the development in old mice from 6 to 10 months of age. Maspin heterozygotes were crossed. Newborn male mice at day 2-day 8 were dissected for anterior and ventral prostate. The number of nodes were counted. Our data showed that maspin KO mice had significant reduced numbers of nodes in both AP and VP than wildtype mice. The KO AP and VP showed increased stromal recruitment and smaller lumen size (see Fig1 &2).

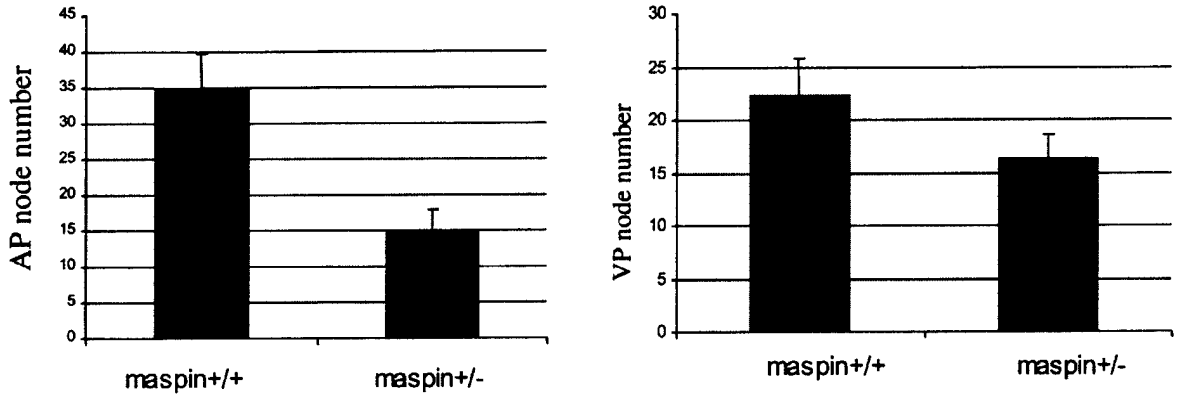

Fig. 1. Morphological analysis of day 8 prostate (AP, VP) in maspin +/+ and maspin+/- mice.

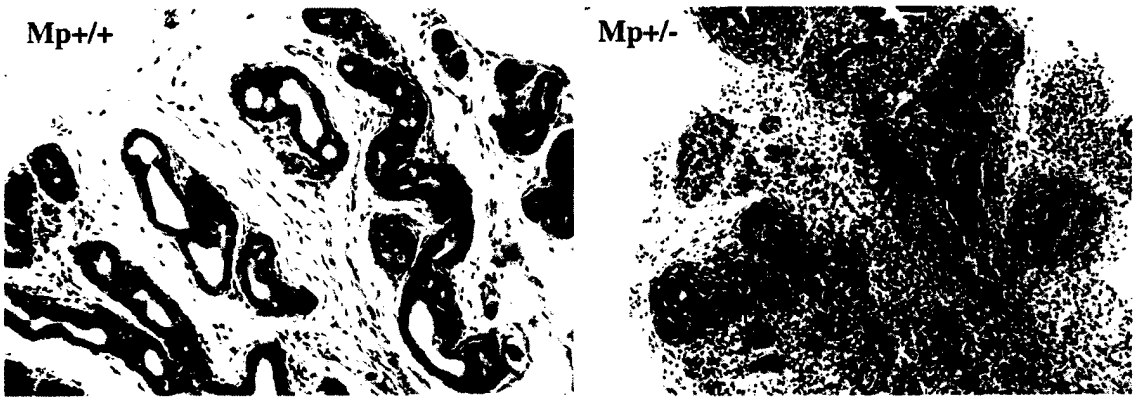

Fig.2 Histology of day 8 Mp+/+ and Mp+/- prostate (AP). Note the increased stroma in Mp+/- AP.

Staining of sections with E-cadherin and ZO-1 revealed changed polarity of epithelial cells in KO AP and VP. We are currently collaborating with Dr. David Rowley to investigate whether this is due to the generation of reactive stroma in maspin KO prostate. For the old prostate, maspin KO mice had increased hyperplasia (PIN) in VP and AP. We are currently carrying out immunostaining for both proliferation and TUNEL assay. These findings indicate that maspin plays a role in normal prostate development. This work clearly will lead to a good publication although we are unable to finish it by the grant deadline. It has laid a good foundation for us to continue to study maspin in prostate in the future.

Key research accomplishments

This study has generated two manuscripts and the study of maspin knockout mice is still in progress.

Reportable outcome

1. Maspin functions as a tumor suppressor by increasing cell adhesion to extracellular matrix in prostate tumor cells. Shaji Abraham, Weiguo Zhang, Norm Greeenberg, and Ming Zhang. J. of Urology, 2003, in press.
2. Maspin inhibits prostate tumor growth in a syngeneic mouse model. Heidi Shi, Longjiang Shao, and Ming Zhang. Manuscript attached.
3. The study of maspin heterozygous knockout mice has generated very exciting data.

Conclusion

Two tasks proposed in the grant were initiated in the first year of proposal. The study of maspin overexpression has established the role of maspin in suppressing prostate tumor development. We have shown that maspin inhibits prostate tumorigenesis by both in vitro and in vivo approaches. This study resulted in one publication and one paper was under review. The project on maspin knockout mice was partially completed. It was affected by the tropical storm. We have recently identified interesting phenotypes in the prostate of maspin heterozygous mice. Continued study will likely generate more information about maspin's role in normal prostate development.

Reference

None

Appendices

1. Manuscript for J.U.
2. Manuscript submitted.

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APPENDIX 1

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MASPIN FUNCTIONS AS A TUMOR SUPPRESSOR BY INCREASING CELL ADHESION TO EXTRACELLULAR MATRIX IN PROSTATE TUMOR CELLS

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ABSTRACT

Purpose: Maspin, a unique member of the serine protease inhibitor family, shows tumor suppressing activity for breast cancer progression and metastasis. Few studies have directly linked maspin function to prostate cancer. We used prostate tumor cells derived from the TRAMP (transgenic adenocarcinoma of mouse prostate) prostate tumor model to study the tumor suppressive function of maspin in prostate cancer.

Materials and Methods: Maspin cDNA was introduced via a retroviral plasmid into TRAMP C2N prostate tumor cells, which are aggressive and invasive in nature. We investigated the tumorigenesis of these stable cell lines in vitro by assessing the growth rate, anchorage independence and adhesion to extracellular matrix proteins such as fibronectin and laminin.

Results: Stable cell lines expressing maspin had decreased tumorigenic potential, as assessed by anchorage independent growth in soft agar assay compared with controls. Maspin stable transfectants showed decreased metastatic potential, as evaluated by modified Boyden chamber assay and increased adhesion to fibronectin and laminin.

Conclusions: Our study confirms that maspin has a tumor suppressive role not only in breast cancer, but also in prostate cancer. The data in this study suggest that maspin can decrease the tumorigenic and metastatic potential of prostate tumors, most probably by remodeling cell-extracellular matrix interactions or triggering extracellular matrix mediated signaling pathways that negatively regulate tumor migration and invasion.

KEY WORDS: prostate, prostatic neoplasms, neoplasm metastasis, cell adhesion, disease progression

Prostate cancer is one of the leading cancer related deaths in men older than 55 years.[1] Essentially a thorough understanding of the disease progression at the molecular level is important for alleviating and achieving an effective cure. In vitro prostatic cells and in vivo animal models serve as good model systems to identify prognostic markers for early disease detection and find therapeutic agents for treatment.[2] We investigated the effect of maspin effect in prostate tumors in vitro in the TRAMP (transgenic adenocarcinoma of mouse prostate) animal model.[3]

Maspin, initially identified from normal mammary epithelial cells, is a unique member of the serine protease inhibitor family that has been found to inhibit breast tumor development.[4] Among the serine protease inhibitor proteins maspin is considered a class II tumor suppressor gene,[5] since the gene is not mutated or deleted, but transcriptionally down-regulated in breast cancer.[6] Several studies have alluded to the tumor suppressive effects of maspin. For example, recombinant maspin protein inhibits breast tumor cell migration and invasion.[7,8] Zhang et al observed the inhibitory effect of maspin on angiogenesis in rat cornea and in a xenograft model.[9] High maspin expression was associated with the absence of lymph node metastasis and better overall survival in oral squamous cell carcinoma.[10] Finally the inhibition of human breast cancer by peroxisome proliferator activated receptor-γ, a differentiation agent, and γ linolenic acid was associated with up-regulation of the maspin gene.[11,12] These studies suggest the significance of maspin for negating tumor development.

Several animal studies done at our laboratory implicate the direct involvement of maspin in the suppression of primary tumor and metastasis.[4,13] When transgenic mice over expressing maspin in mammary epithelial cells under the control of mammary specific whey acidic protein promoter[14] were crossed with a strain of oncogenic whey acidic protein-simian virus 40 T antigen mice, the resulting bitransgenic mice showed inhibited tumor growth and metastasis.[13] In addition, when we tested the tumor suppressing activity of maspin in a TM40D syngeneic model in which TM40D cells or maspin transfectants were implanted back into a mammary gland, we found that the tumor growth and metastasis rates were significantly decreased in maspin transfectants.[15] These data demonstrate that maspin blocks primary tumor growth and metastasis in immunocompetent animals.[13,15]

Despite much data supporting the tumor suppressing role of maspin in breast cancer few studies have directly linked maspin function in prostate cancer. For instance, maspin gene expression is down-regulated in prostate cancer cells[16] as well as in clinical prostate cancer specimens.[17] Recombinant maspin made in vitro inhibits prostatic cell migration.[8] However, Umekita et al noted that rat maspin did not inhibit prostate tumor progression in rat derived cells.[18] Since in the TRAMP prostate animal model tumors develop that closely resemble human prostatic disease,[3] we examined the role of maspin in mouse prostate tumor cells. Thus, we introduced the maspin gene into C2N TRAMP prostate tumor cells, a highly tumorigenic and invasive cell line,[19] by a retrovirus approach.

In this study we investigated the consequence of maspin expression in TRAMP cell lines as a prelude to elucidating its function in vivo in prostate tumors. In particular we were interested in identifying whether maspin could decrease the tumorigenic and metastatic potential of C2N tumor cells. We

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report that maspin can inhibit tumor invasion and increase cell-extracellular matrix (ECM) adhesion of invasive C2N tumor cells. Thus, maspin functions as a tumor suppressor, probably by remodeling cell-ECM interaction, in this prostate cancer model.

MATERIALS AND METHODS

Cell lines and tissue culture conditions. C2N cells were grown in Dulbecco's modified Eagle's medium (DMEM) without Na pyruvate supplemented with 50% heat inactivated fetal bovine serum and 50% Nu serum (Becton Dickinson, Sunnyvale, California), 0.0001 M. dihydrotestosterone, 0.05% insulin (Sigma Chemical Co., St. Louis, Missouri), and 0.05% penicillin and streptomycin at 37C in 5% CO_2. Human embryonic 293T cells were grown in DMEM containing 10% heat inactivated fetal bovine serum at 37C in 5% CO_2.

Plasmid constructs. The retroviral plasmid pS2-green fluorescent protein (GFP) was constructed by inserting GFP-zeocin from pTracer-CMV (Invitrogen, Carlsbad, California) into pS2, a retroviral plasmid containing the 5′ and 3′ long terminal repeat regions.[20] The 1.2 kb human maspin cDNA was amplified by polymerase chain reaction (PCR), filled in with Klenow enzyme and cloned into pS2-GFP vector at the Apa I site. The resultant plasmid construct was named pS2-GFP maspin.

Establishment and selection of stable transfectants. The retroviral plasmid constructs pS2-GFP or pS2-GFP maspin (10 μg.) were transfected into 293 T cells with 10 μg. pCL-Eco plasmid using fugene reagent, as described by Naviaux et al.[21] After 24 hours C2N TRAMP cells were grown in the presence of polybrene (10 mg./ml) and viral particles for 3 hours. Virus containing medium was then replaced by fresh medium. The cells were allowed to grow for a week and then GFP or GFP-maspin stable transfectants were selected in growth medium containing 100 μg./ml. zeocin for 2 weeks. Stable transfectants were further sorted by an Altra (Beckman-Coulter, Inc., Chaska, Minnesota) flow cytometry and pools of GFP expressing cells were obtained. Control GFP expressing cells were confirmed for green fluorescence by microscopy using a model DMRB (Leica Mikroskope und Systeme GmbH, Wetzlar, Germany). GFP-maspin expressing cells were plated into 96-well plates and subclones of stable transfectants were isolated. Reverse transcriptase (RT)-PCR, Western blotting and immunostaining experiments confirmed the presence of the GFP-maspin gene in the stable transfectants.

Western blot analysis. The cells were lysed in RIPA buffer containing protease inhibitors (aprotinin, leupeptin and phenylmethylsulfonyl fluoride) and 50 μg. protein were size fractionated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. The proteins were transferred onto a polyvinylidine difluoride membrane (BioRad Laboratories, Hercules, California), blocked in 5% nonfat dry milk overnight and immunoblotted with maspin antibodies (Pharmingen, San Diego, California). With the signal amplified by secondary antibody linked to horseradish peroxidase it was detected by an enhanced chemiluminescence kit (Pierce, Rockford, Illinois). The blots were stripped and re-probed with β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, California) to confirm equal sample loading.

Cell proliferation assay. Stable GFP or GFP-maspin C2N transfectants were plated at a density of 1×10^3 cells per well in a 24-well plate in triplicate. At indicated time points of 1, 2, 4, 6 and 8 days growth was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, Wisconsin) assay according to the manufacturer protocol. Absorbance was recorded at 420 nm. using an MRX (Dynex Technologies, Inc., Chantilly, Virginia) model microplate reader.

Cell adhesion assay. Stable GFP and GFP-maspin transfectants were harvested, washed with 1 × phosphate buffered saline, re-suspended in serum free medium with bovine serum albumin (10 mg./ml.) and plated at a density of 1×10^3 cells per well in a 24-well plate pre-coated with laminin (25 μg./ml.) or fibronectin (25 μg./ml.). The cells were incubated at 37C and 5% CO_2 for 2 hours. Nonadherent cells were removed by aspiration and adherent cells were washed 3 times with 1 × phosphate buffered saline. Total cell associated protein was determined by adding 200 μl. BCA (Pierce) working solution directly to the wells, incubating at 60C for 2 hours and lysing with 0.1% sodium dodecyl sulfate. The absorbance of each well was determined at 562 nm. in an MRX model microplate reader. Experiments were repeated 3 times in triplicate.

Colony formation in soft agar. For this assay the bottom layer contained 0.6% agarose in DMEM, while the top layer contained 0.3% agarose. Stable transfectants were seeded at a density of 6,000 cells per well in a 6-well plate in triplicate. The plates were incubated at 37C with 5% CO_2 for 3 weeks and then stained with p-iodonitrotetrazolium violet (1 mg./ml.) for 16 hours at 37C. Colonies greater than 1 mm. were counted under an inverted microscope (Leica). Colony formation was assessed in 3 independent experiments.

Cell invasion assay. Cell invasion was performed in a modified Boyden chamber assay. Briefly, the inner chamber of Millicell (Millipore, Bedford, Massachusetts) polycarbonate filters (pore size 8 μm.) coated with 2.5 mM. Matrigel in serum-free DMEM was dried overnight at room temperature under ultraviolet light. The pre-coated filters were placed in 24-well plate that formed the lower chamber and the lower chamber was filled with DMEM containing 10% serum. Cells were seeded at a density of 6×10^4 cells per well and incubated at 37C for 4 hours with 5% CO_2. After incubation cells remaining in the inner chamber were removed with a cotton swab. Cells on the outer surface of the inner chamber were fixed with 3% glutaraldehyde solution and stained with hematoxylin and eosin. Cells that penetrated the inner chamber were counted using an IX70 (Olympus Optical Corp., Melville, New York) phase contrast microscope. Experiments were performed in triplicate and repeated twice.

Statistical analysis. All statistical significance was determined by Student's t test with $p < 0.05$ considered statistically significant. Graphic presentation of the data was done using Excel (Microsoft, Seattle, Washington) spread sheet software.

RESULTS

Stable expression of maspin in C2N TRAMP cells. C2N prostate tumor cells are highly invasive and tumorigenically isolated from a primary prostate tumor in TRAMP mice. In this cell line maspin expression was not detected by RT-PCR and Western blot analysis (data not shown). To re-express maspin we transfected retroviral vectors pS2-GFP or pS2-GFP maspin into C2N cells. Zeocin resistant cells were sorted by flow cytometry and pools of GFP expressing cells were isolated (data not shown). Stable transfectants expressing variable levels of maspin were subcloned. A total of 52 single cell clones expressing variable levels of maspin were identified and representative clones were further characterized. They were examined for maspin mRNA expression by RT-PCR and for protein by immunostaining (data not shown). The level of maspin expression was quantitated by Western blot analysis. Figure 1 shows that 1 representative clone (clone 2) expressed a low level of maspin and 2 (clone 25 and 46) expressed medium and high level of maspin, respectively, while the control pS2-GFP clone had no detectable maspin expression. These clones were used for subsequent experiments.

Effects of maspin on cell proliferation in C2N tramp cells.

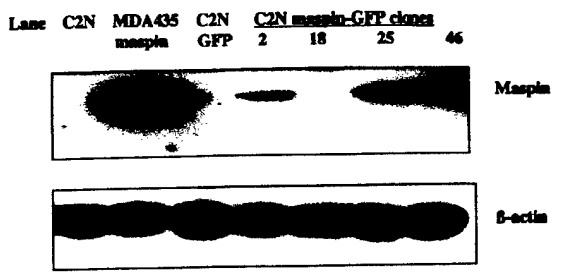

FIG. 1. Western blot analysis of C2N maspin-GFP stable transfectants. Cell lysates (50 μg.) from C2N stable transfectants were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred onto polyvinylidine difluoride membrane and probed with mouse monoclonal antibody to human maspin. Band (42 kDa.) was identified in several clones. Blot was stripped and probed with β-actin antibodies for equal sample loading.

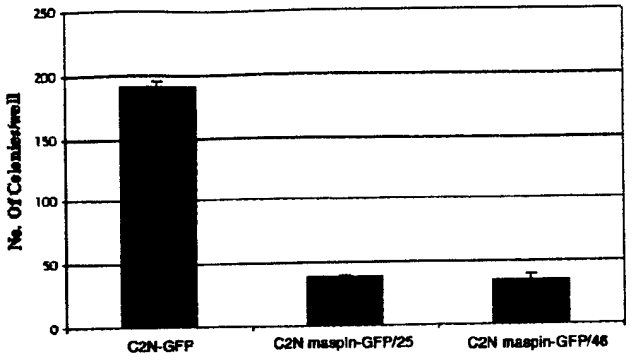

FIG. 3. Anchorage independent growth of maspin expressing C2N cells. Clones indicated were plated in soft agar in triplicate in 6-well plate and incubated at 37C in 5% CO_2 for 3 weeks. Cells were stained with p-nitrotetrazolium violet for 16 hours at 37C, counted and photographed using dissecting microscope linked to Leica camera. Each sample was assayed in triplicate and experiment was repeated twice. Error bars represent ± SD.

To investigate the biological effects of maspin on invasive C2N cells we tested the growth rates of stable transfectants. When cells were grown in log phase for 2 to 5.2 days, a striking variation in the growth pattern was observed among stable transfectants on MTS assay. Cells reached confluence by days 6 to 7, after which the assay was stopped. Indeed, stable transfectants expressing maspin showed a lower growth rate than control transfectants (pS2-GFP) and the parental cell line. Moreover, the level of maspin expression determined growth inhibition in a dose dependent manner. Clone 2 expressing low levels of maspin had lower growth inhibition than high maspin expressing clones 25 and 46. The vector-only control clone had a growth pattern similar to that of parental C2N cells (fig. 2). These data indicate that maspin was responsible for growth suppression.

Effect of maspin on anchorage independent growth of C2N cells. We determined the antimetastatic potential of maspin in vitro by soft agar assay. After plating 6×10^3 cells in triplicate in soft agar the number of colonies formed and colony size were analyzed at the end of 3 weeks. Colonies greater than 1 mm. were scored. Figure 3 shows that GFP-vector only stable transfectants demonstrated a higher capability of colony formation in soft agar, while clones over expressing maspin showed a lower capability of colony formation ($p < 0.01$). Moreover, plating more cells or increasing incubation period did not increase the colonies of clones 25 and 46 (data not shown). These data indicate that maspin expression decreased the anchorage independent growth capacity of C2N cells.

Increased adhesion of maspin transfectants to ECM components fibronectin and laminin. Cell adhesion to ECM is a preliminary step involved in invasion and metastasis. ECM consists of a composite pool of matrix proteins with laminin and fibronectin as its major components. Therefore, we investigated the ability of stable transfectants to adhere to the ECM protein fibronectin or laminin. On adhesion assays maspin stable transfectants showed a 60% to 70% increase in binding to laminin and fibronectin compared with vector-only control transfectants (fig. 4). Such changes in adhesion to laminin and fibronectin were statistically significant ($p < 0.02$ and < 0.01, respectively). These data demonstrate that maspin can indeed increase the ability of cells to adhere to ECM molecules, although in a dose independent manner.

Decreased chemo-invasion of maspin transfectants in vitro. The metastatic potential of tumors depends on the ability of the tumor cells to invade through the basement membrane and migrate to distant sites. We examined the ability of maspin transfectants to penetrate Matrigel using the modified Boyden chamber assay. Significant differences in cell invasion were observed in vector-only transfectants and

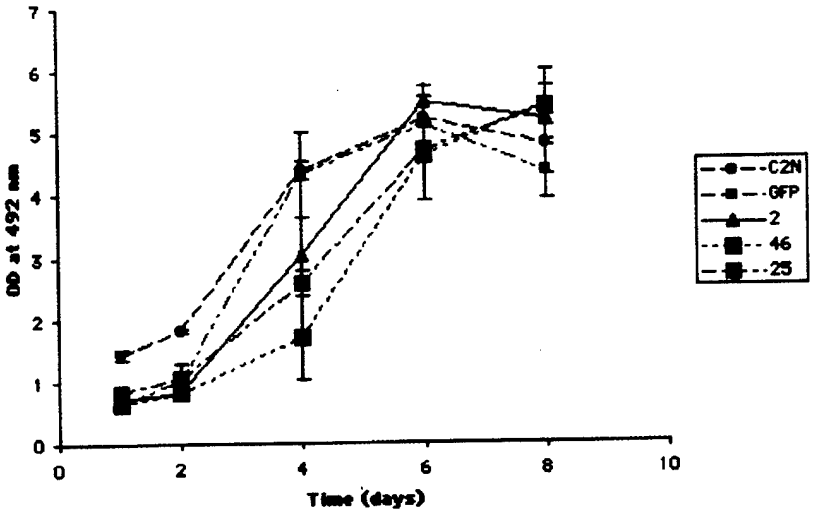

FIG. 2. Growth inhibition of C2N maspin-GFP expressing clones. Cells were seeded at 1×10^3 in 24-well plate and growth was measured by MTS assay on days indicated. Error bars represent ± SD of 3 experiments. *OD*, optical density.

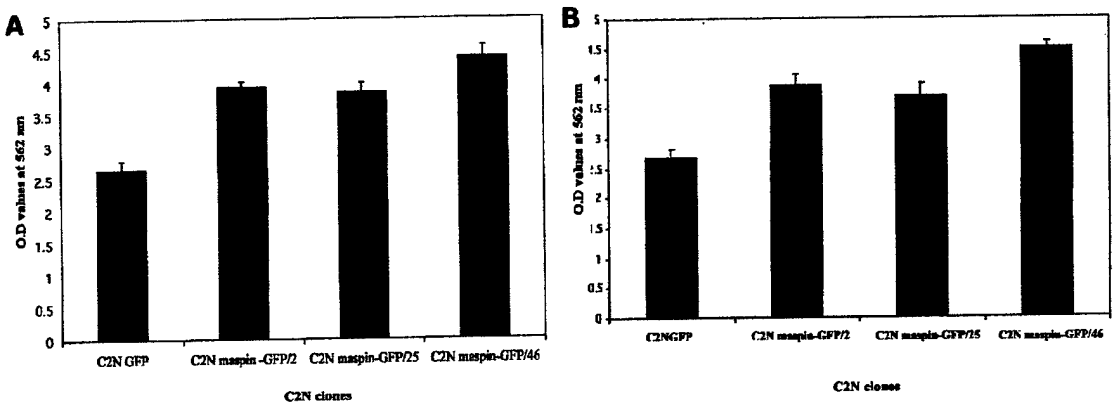

FIG. 4. Cell adhesion of stable transfectants to matrix proteins. *A*, adhesion assay of stable transfectants to fibronectin. Cells were seeded onto wells coated with 25 μg./ml. fibronectin and incubated at 37C for 2 hours. Cell density was measured by protein estimation using BCA reagent. *B*, adhesion assay of stable transfectants to laminin. Cells were seeded onto wells coated with 25 μg./ml. laminin and incubated at 37C for 2 hours. Cell density was measured by protein estimation using BCA reagent. Error bars represent ± SEM of 3 experiments done in triplicate ($p < 0.05$). *O.D.*, optical density.

maspin transfectants. The rate of invasion was significantly higher in vector-only transfectants than in stable transfectants expressing maspin (fig. 5). Clone 2 expressing a lower maspin level had a higher rate of invasion, similar to vector-only transfectants. A dose dependent decrease in invasion was observed ($p < 0.05$). These data show that maspin expressed in prostate tumor cells could actively inhibit the ability of their invasion through Matrigel.

DISCUSSION

In the current study the maspin gene was expressed endogenously in highly invasive C2N TRAMP cells by retrovirus infection. Clones 2, 25 and 46 were arbitrarily selected based on maspin expression (low, medium or high) at the protein level. Our data indicated that cells expressing maspin were less invasive in Matrigel and showed less ability to grow in 3 dimensions in soft agar assay than control cells. Maspin expression also seemed to increase cell-ECM contact as assessed. Together these findings suggest that the tumor suppressive effect of maspin in prostate tumors is most probably achieved through cell-ECM interactions.

Tumor invasion and metastasis represent a complex process involving protease degradation of the basement membrane, migration and invasion of tumor cells.[22] A key question is how maspin exerts its tumor suppressing function in cancer. Sheng et al presented evidence in which maspin interacted with fibrinogen associated tissue plasminogen activator and with cell surface associated urokinase plasminogen activator.[23] Seftor et al showed that maspin suppressed invasive phenotypes of human breast cancer cells by modulating their integrin expression.[7] Recently Blacque and Worrall provided new evidence that maspin interacts with types I and III collagen directly, suggesting that maspin could exert its anti-invasion and antimetastatic function by modulating cell adhesion with the extracellular matrix.[24] Two major components of ECM in prostate and mammary glands are laminin and fibronectin. To test whether maspin expressing prostate tumor cells had different cell adhesion affinity to laminin and fibronectin than those without maspin or lower maspin expressing cells we performed a cell adhesion assay. In the presence of either matrix molecule maspin stable transfectants adhered more to matrix components than control cells. This result is consistent with a previous study in which MDA 231 breast tumor cells treated with maspin had increased integrin activity, which resulted in increased cell adhesion to fibronectin.[7] Moreover, Ngamkitidechakul et al noted that corneal stromal cells increased adhesion to fibronectin and laminin in the presence of exogenous maspin.[25] The increased adhesion of maspin expressing C2N cells would likely make them more attached to the ECM, thus, preventing tumor cells from migrating freely through the basement membrane or triggering ECM medi-

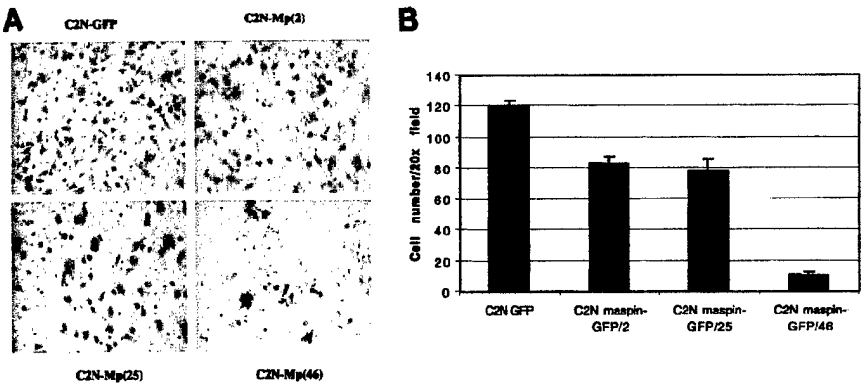

FIG. 5. Decreased cell invasion of C2N maspin stable transfectants. *A*, representative view of cells on chamber membrane. Cells were seeded onto filters coated Matrigel in modified Boyden chamber assay and incubated at 37C for 4 hours. Invaded cells were stained and counted under microscope. H & E, reduced from ×400. *B*, analysis of cell invasion assay. Error bars represent ± SD of 3 independent experiments (2-tailed t test $p < 0.05$).

ated signaling pathways that negatively regulate migration. In addition, using the Boyden chamber system we evaluated the effect of maspin on C2N cell invasion. Figure 5 shows that maspin expressing clones were greatly inhibited in their ability to invade through the matrix membrane. The difference was highly significant and reproducible. Thus, our data conclusively demonstrate that maspin can inhibit C2N tumor invasion in a fashion similar to that observed in breast tumors. Blocking tumor cell invasion and migration is an essential step for antimetastatic therapy because it limits tumor intravasation and extravasation.

CONCLUSIONS

Our study using TRAMP cells from the TRAMP mouse, an excellent model for prostate cancer, confirms the role of maspin as a tumor suppressor in prostate cancer. This study demonstrates decreased cell invasion in the presence of maspin, a phenomenon of paramount importance in metastasis. In fact, the tumor suppressive role of maspin is most likely achieved by preventing tumor cell invasion through the basement membrane and, thereby, preventing metastasis. Hence, the data further suggest that maspin can be a therapeutic target not only for breast cancer, but also for prostate cancer.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S. and Wingo, P. A.: Cancer statistics, 1998. CA Cancer J Clin, **48:** 6, 1998
2. Navone, N. M., Labate, M. E., Troncoso, P., Pisters, L. L., Conti, C. J., von Eschenbach, A. C. et al: p53 Mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. J Urol, **161:** 304, 1999
3. Greenberg, N. M., DeMayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O. et al: Prostate cancer in a transgenic mouse. Proc Natl Acad Sci USA, **92:** 3439, 1995
4. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S. et al: Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science, **263:** 526, 1994
5. Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G. et al: RNA genetics of breast cancer: maspin as paradigm. Cold Spring Harb Symp Quant Biol, **59:** 537, 1994
6. Zhang, M., Maass, N., Magit, D. and Sager, R.: Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. Cell Growth Differ, **8:** 179, 1997
7. Seftor, R. E., Seftor, E. A., Sheng, S., Pemberton, P. A., Sager, R. and Hendrix, M. J.: Maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res, **58:** 5681, 1998
8. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J. and Sager, R.: Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci USA, **93:** 11669, 1996
9. Zhang, M., Volpert, O., Shi, Y. H. and Bouck, N.: Maspin is an angiogenesis inhibitor. Nat Med, **6:** 196, 2000
10. Xia, W., Lau, Y. K., Hu, M. C., Li, L., Johnston, D. A., Sheng, S. et al: High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. Oncogene, **19:** 2398, 2000
11. Jiang, W. G., Hiscox, S., Horrobin, D. F., Bryce, R. P. and Mansel, R. E.: Gamma linolenic acid regulates expression of maspin and the motility of cancer cells. Biochem Biophys Res Commun, **237:** 639, 1997
12. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M. et al: Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell, **1:** 465, 1998
13. Zhang, M., Shi, Y., Magit, D., Furth, P. A. and Sager, R.: Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene, **19:** 6053, 2000
14. Zhang, M., Magit, D., Botteri, F., Shi, Y., He, K., Li, M. et al: Maspin plays an important role in mammary gland development. Dev Biol, **215:** 278, 1999
15. Shi, H. Y., Zhang, W., Liang, R., Abraham, S., Kittrell, F. S., Medina, D. et al: Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res, **61:** 6945, 2001
16. Zhang, M., Magit, D. and Sager, R.: Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci USA, **94:** 5673, 1997
17. Machtens, S., Serth, J., Bokemeyer, C., Bathke, W., Minssen, A., Kollmannsberger, C. et al: Expression of the p53 and Maspin protein in primary prostate cancer: correlation with clinical features. Int J Cancer, **95:** 337, 2001
18. Umekita, Y., Hiipakka, R. A. and Liao, S.: Rat and human maspins: structures, metastatic suppressor activity and mutation in prostate cancer cells. Cancer Lett, **113:** 87, 1997
19. Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C. and Greenberg, N. M.: Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res, **57:** 3325, 1997
20. Armentano, D., Yu, S. F., Kantoff, P. W., von Ruden, T., Anderson, W. F. and Gilboa, E.: Effect of internal viral sequences on the utility of retroviral vectors. J Virol, **61:** 1647, 1987
21. Naviaux, R. K., Costanzi, E., Haas, M. and Verma, I. M.: The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J Virol, **70:** 5701, 1996
22. Liotta, L. A., Steeg, P. S. and Stetler-Stevenson, W. G.: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, **64:** 327, 1991
23. Sheng, S., Truong, B., Fredrickson, D., Wu, R., Pardee, A. B. and Sager, R.: Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. Proc Natl Acad Sci USA, **95:** 499, 1998
24. Blacque, O. E. and Worrall, D. M.: Evidence for a direct interaction between the tumor suppressor serpin maspin, and types I and III collagen. J Biol Chem, **277:** 10783, 2002
25. Ngamkitidechakul, C., Burke, J. M., O'Brien, W. J. and Twining, S. S.: Maspin: synthesis by human cornea and regulation of in vitro stromal cell adhesion to extracellular matrix. Invest Ophthalmol Vis Sci, **42:** 3135, 2001

APPENDIX 2

Maspin inhibits prostate tumor growth in a syngeneic mouse model

(Running title: Maspin inhibits prostate tumor progression)

(Key words: maspin, prostate tumor, invasion and metastasis, TRAMP, and angiogenesis)

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Abstract

Background. Maspin, a unique member of serpin family exhibits tumor-suppressing activity in breast cancer progression and metastasis. Few studies have directly linked maspin's function in prostate cancer. We have used prostate tumor cells derived from the TRAMP prostate tumor model to study maspin's tumor suppressive function in a syngeneic model and in cell culture.

Methods. The maspin cDNA was introduced to TRAMP C2N prostate tumor cells, and stable transfectants were inoculated to syngeneic C57BL/6 mice for analysing maspin's effect in vivo. A 3D-culture system was exploited to study maspin's effect on tumor cell apoptosis. Condition medium collected from maspin expressing cells was used to determine the effect of secreted maspin on endothelial migration.

Results. Maspin overexpressing tumor cells were inhibited in their abilities to form palpable tumor foci in syngeneic mice. We determined lung metastasis rate for C2N tumor model. In a 3D-cell culture system, maspin expressing tumor cells displayed significant higher apoptosis rate as compared to control tumor cells. Maspin expressing cells also secreted sufficient amount of maspin that inhibited endothelial cell migration in Boyden chamber assay.

Conclusion. Our study confirms that maspin inhibits prostate tumor progression in syngeneic mouse model in vivo. Such inhibition is likely through the increased apoptosis in tumor cells and the decreased angiogenesis.

Introduction

Prostate cancer is one of the leading cancer related deaths in men over the age of 55 (1). Identification of genes involved in prostate cancer and understanding of their roles in tumor progression is important for cancer prevention and therapy. *Several studies utilizing* prostatic cells in vitro and *in vivo* animal models are reported which serve as good model systems in identifying prognostic markers in early disease detection, and in finding therapeutic agents for treatment (2). One animal model is the TRAMP (transgenic adenocarcinoma of mouse prostate) animal model (3). In this model, SV 40 early region (T, t antigen) was targeted to the epithelium of the mouse prostate by a prostate specific promoter. TRAMP mice develops prostate tumors, which closely mimic human prostatic disease. Prostatic epithelial cell lines derived from tumors of TRAMP mice were established (4). Some of them were highly tumorigenic and invasive carcinoma and can be used as an *in vitro* system to assess cancer therapy.

Maspin, initially identified from normal mammary epithelial cells, is a unique member of the serpin (serine protease inhibitor) family, and found to inhibit breast tumor development (5). Among the serpin proteins, maspin is considered a class II tumor suppressor gene, since the gene is not mutated nor deleted, but is transcriptionally down regulated in breast and prostate cancers (6). Several studies have alluded to the tumor suppressive effects of maspin. For example, recombinant maspin protein inhibits breast tumor cell migration and invasion (7, 8). Angiogenesis was inhibited in rat cornea and in a xenograft model by maspin (9). High level of maspin expression was associated with the absence of lymph node metastasis and with better overall survival in oral squamous cell carcinoma (10). Recent clinical studies have linked

maspin's tumor suppression function with better prognosis in prostate cancer (11, 12), suggesting the significance of maspin in negating prostate tumor development.

To study maspin function in prostate tumor, we introduced maspin cDNA into C2N TRAMP prostate tumor cells, a highly tumorogenic and invasive cell line (4), by a retrovirus approach. In a recent study, we investigated the effect of maspin expression in TRAMP tumor cell adhesion and invasion (13). The data indicated that maspin could inhibit tumor invasion and increase cell-ECM (extracellular matrix) adhesion of invasive C2N tumor cells. To further test maspin's tumor inhibitory function in vivo, we implanted maspin expressing tumor cells and the control tumor cells into the syngeneic mice. Here, we report our finding that the presence of maspin inhibits the prostate tumor progression in implantation model. Furthermore, we demonstrated that this inhibition is due to the increased apoptosis and an inhibition of angiogenesis by the secreted maspin from C2N maspin transfectants.

Materials and methods

Cell lines and tissue culture conditions

C2N cells were obtained from Dr. Norman Greenberg, grown in DMEM (w/o Na pyruvate) medium supplemented with 50% heat inactivated fetal bovine serum, 50% Nu serum (Beckton Dickson Lab, Inc., CA), 0.0001M dihydrotestosterone (Sigma, MO), 0.05% insulin (Sigma, MO) and 0.05% Penicillin and streptomycin at 37 ^{o}C and 5% CO_2. Stable clones (C2N, C2N-46, C2N-25) which contain empty vector or maspin were established as described previously (13). Zeocin was purchased from Invitrogen Co., CA.

Implantation of transfectants to C57BL/6 mice

C57BL/6 mice were initially purchased from Harlan, Inc. and bred in our animal facility to generate offspring males of 8-week age. In group 1 and 2, the brother pairs were divided for the implantation of maspin clones or control clones. Tumor initiation was monitored twice a week by palpation. Tumor volume was calculated using the formula: length x width^2 /2 (14). Tumor growth rate was monitored every two days by caliper measurement.

C2N-46 maspin cells and C2N control cells were used in the implantation. All tumor cells were grown to 80% of confluence before being harvested for cell counting. Total 1x10^6 maspin transfectant cells or equal number of control cells were suspended in 100 μl of HEPES-buffered salt solution (HBSS) and were mixed with 100 μl of Matrigel (Collaborative Research, MA). The mixtures were injected subcultaneously into the dorsal back of 8 week old male C57BL/6 mice (one inoculation site per animal). For group 1, 12 mice were used for C2N-46 maspin transfectants and nine for controls. In a separate experiment (group 2), 13 mice were used for C2N-46 and 12 mice for C2N. For the C2N inoculated group, mice were sacrificed when the primary tumors grew to about 1.5 cm in diameter. For the C2N-46 inoculated group, all mice were sacrificed six months after inoculation. No palpable tumor was observed in any mice within this group.

Tumor histology and immunohistochemistry

Prostate tumors were fixed in 10% neutral formalin buffer, embedded in paraffin, and sectioned at 5 μm. Sections were stained by H&E. Vessel density was evaluated by immunostaining using CD31 antibody. Tissue sections were treated with proteinase K (10 μg/ml) for 10 minutes at 37 °C and quenched with 0.03% of H_2O_2 in PBS for 30 minutes

at room temperature. The slides were blocked with 10% normal horse serum for 1 hour before they were treated with the primary antibody anti-CD31 monoclonal antibody (PharMingen, CA) was used at 1:50 dilution at 4 ^{0}C overnight. Slides were then rinsed and incubated with an avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories, CA), followed by DAB color development (DAB kit, Zymed, Inc., CA).

For lung metastasis analysis, lung tissues from all C2N inoculated mice (19 mice) were collected at the time of euthanasia. Fixed lung tissues were cut into 5 μm sections. Two sections separated by 100 μm were stained with hematoxylin/eosin and examined for the presence of metastases.

Three dimension cell culture system and TUNEL assay

For spheroid 3D cell culture, the subcloned cells were seeded in non-precoated culture dishes and cultured in 37C, 5% CO2 incubator with shaking for 3 days (15). As a result, cells did not attach to the substratum but simply grew and formed small 3D colonies. They were then transferred to Matrigel-covered petri dishes and kept in culture for one week.

For tumor section TUNEL assay, tumor samples were embedded and sectioned to 5 μM slides. In situ TUNEL assay was carried for sections as described previously. The number of prostate cancer cells undergoing apoptosis was determined by counting the number of apoptotic cells in the section. Four fields were counted for each tumor sample and the numbers were averaged.

Migration assay

Migration assay were carried out by a modified method as described previously (9). HUVEC cells ($1.2x10^6$ cells/ml) were metabolically labeled by ^{35}S –leu and met amino acids (Amersham, Inc., NJ) for 1 hour and plated in serum-free basal media supplemented with 0.1%BSA in modified Boyden chamber (50 µl). Test substances, which include condition medium, maspin antibody or preimmune serum were added to the top chamber (volume 5 µl). The radioactive labeled cells that were migrated to the bottom chamber were counted in a scintillation counter.

Western blot analysis: Condition medium from C2N-46 and C2N cell culture were collected and concentrated 20 fold by centrifugation using centricon tube (Millipore, Inc., MI). The concentrated proteins (50 µl) were size-fractionated on a 10% SDS-PAGE gel. Bacterial recombinant rMaspin protein (1µg) was loaded on the gel as a positive control. The proteins were transferred onto a PVDF membrane (Bio-rad, CA), blocked in 5% non-fat dry milk overnight, immunoblotted with maspin antibody (AbS4A) and, the signal amplified by secondary antibody linked to horse-radish peroxidase was detected by enhanced chemiluminiscence (Pierce, Rockford, IL) kit.

Statistical Analysis: All statistical significance was determined by student's t-test (two-sided analysis) and Chi-square analysis. A p-value of < 0.05 was considered statistically significant. Graphic presentation of the data was done using Excel spread sheet software (Microsoft, Seattle, WA).

Results:

Maspin inhibits prostate tumor growth in a syngeneic prostate tumor model

The C2N prostate tumor cells were isolated from a primary prostate tumor in TRAMP mice. This cell strain is highly invasive and tumorigenic. We have previously transfected maspin gene into C2N cells and established stable transfectants expressing variable levels of maspin. In the in vitro experiment, maspin transfectants were found to inhibit the soft agar colony formation, and increase tumor cell adhesion to ECM(13). To study maspin's role in vivo, we implanted maspin transfectants and control C2N tumor cells into the syngeneic C57BL/6 mouse to observe tumor growth in vivo. Two clones expressed medium and high level of maspin (clone #25 and #46)(13), and control clone which had no detectable maspin expression were used for subcutaneous implantation in C57BL6 mice. Two separate experiments were carried out using C2N and C2N-46 cells for implantation. The first group implanted 9 and 12 mice with C2N, and C2N-46 cells respectively. Eight of the nine C2N implanted mice developed palpable tumors. In the second group, which consists of 12 C2N control mice and 13 C2N-46 implanted mice, eleven of the 12 C2N control mice developed tumors. Overall, control tumor cells grew out palpable tumors with high frequency (90.5%). It took an average of 57 days for C2N tumors to grow from the size of 0.1 cm to 1.5 cm in diameter. However, none of our maspin transfectants had developed any palpable tumors in the syngeneic mice at time period comparable to that of C2N transfectants (Table 1). We have extended observation time for 6 months for C2N-46 implanted mice. No palpable tumors were observed in any C2N-46 implanted mice. The difference in tumor forming ability between C2N and C2N maspin transfectants was statistically significant ($P<0.001$, Chi-square). We also carried out additional implantation experiment (10 mice for C2N-25, 5 mice for C2N control) using C2N-25 cells, and we did not observe any tumor

formation for C2N-25 cells in a period of five months (data not shown). These data indicate that maspin inhibits prostate tumor progression in vivo.

Histology analysis of C2N derived tumor samples confirmed the presence of adenocarcinoma (Fig.1a,b). The tumors appeared to be very invasive. In general, the presence of tumor encapsulation was associated with better prognosis. C2N tumor sections lacked tumor encapsulation (Fig.1b). They contained a high microvessel density as indicated by CD31 immunostaining (Fig.1c), indicating that the growth of C2N tumors was highly dependent on the availability of tumor vasculatures (Fig.1B). Furthermore, we determined the metastasis frequency and the time course for C2N tumors in this syngeneic mouse model. Lung tissues from mice bearing C2N tumors were collected and sectioned for microscopic analysis. Two serial sections separated 100 μm were selected to score for micrometastatic tumor foci under high power microscope (Fig.1d). As shown in Table 1, 42.1% (8 out 19) control mice developed lung metastasis.

Effects of maspin on tumor cell apoptosis in 3D spheroid

The maspin expressing transfectants had very limited growth inhibitory phenotype when they were grown in the presence of growth factors in cell culture(13). This partial block on proliferation does not explain why there is a complete suppression of tumor growth in vivo. To determine other factors which may contribute to maspin's tumor suppression property, we utilized a tumor spheroid 3D-culture system. We grew the control C2N cells and C2N-46 maspin expressing cells in 3D-tumor spheroids. These spheroids were harvested, embedded in paraffin, and sectioned to 5 μm slides for H&E histology (Fig.2). As shown in Fig.2, C2N and C2N-46 tumor cells both formed 3D tumor spheroid, and H&E histology showed that tumor cell

morphology was similar to that of the C2N tumors in mice (Fig.1a,b). We examined sections of tumor spheroids for apoptosis rate by the TUNEL assay (Fig.3). The TUNEL assay revealed that C2N-46 tumor cells had a significant higher rate of apoptosis than that of C2N control (Fig.3C). This data suggest maspin induced apoptosis may represent a major contributing factor for the inhibition of prostate tumor growth in vivo.

Serection of maspin by C2N-maspin transfectants and the inhibition of endothelial cell migration by maspin

Another important factor for tumor growth in vivo is that tumor cells require vasculature network to support their growth. Since we have demonstrated that maspin is an angiogenesis inhibitor in other system, we determine whether C2N-46 maspin transfectants secret maspin to inhibit angiogenesis. We cultured C2N-46 and C2N cells and collected conditional medium from both cell cultures. Western blot analysis demonstrated that C2N-46 cells were capable of secreting maspin protein to the conditional medium (Fig.4A).

To determine whether the secreted maspin from C2N-46 is able to inhibit angiogenesis, we examined the ability of the C2N-46 condition medium to affect human umbical endothelial cell (HUVEC) migration by a modified Boyden chamber assay. In this assay, HUVEC cells were metabolically labeled, placed on the upper chamber, and were subjected to the treatment of condition medium from C2N-46 cells or C2N control cells (Fig.4B). Significant differences in cell migration were observed between control and C2N-46 medium treated samples ($p<0.003$). To verify the specificity, maspin-blocking antibody was used in the migration assay. Addition of maspin antibody blocked maspin's inhibitory effect on migration, while control preserum had no

adverse effect (Fig.4B). These data indicate that C2N-46 transfectants expressed sufficient amount of maspin. These maspin proteins were effectively secreted and acted to inhibit the endothelial cell migration.

Discussion

The tumor forming ability in vivo depends on both the properties of tumor cells themselves and the availability of vascular structure to supplement the nutrient for the growth of tumor cells. This is demonstrated in this study in which maspin expressing C2N prostate tumor cells are completely inhibited in their ability to form tumor foci in vivo. It is noted that when C2N-46 maspin transfectants and C2N control cells are both grown in the presence of serum minor difference in cell growth was observed between these two groups of tumor cells. We demonstrated that the major difference lied in the different rate of apoptosis when tumor cells were cultured in the 3D cell culture system. Maspin expressing tumor cells underwent significant higher rate of apoptosis. In addition, C2N-46 maspin cells secreted sufficient amount of maspin which directly inhibited the endothelial cell migration, thus the tumor angiogenesis. These properties explain why the presence of maspin completely inhibits prostate tumor progression in this syngeneic tumor model.

The role of maspin in tumor growth inhibition has been mostly demonstrated in breast cancer models in vivo. Transgenic expression of maspin in mammary gland was shown to inhibit breast tumor progression in a SV40 T antigen induced tumor model (16). Additionally, we utilized a syngeneic mammary tumor model in which maspin was introduced to mammary tumor cells for stable expression. Significant inhibition of both primary tumor growth and metastasis was observed in maspin transfectants as compared to the control(17). To explore whether maspin

plays a similar role in prostate tumor, we carried out this study in which maspin gene was introduced into prostate tumor cells and the transfectants were implanted to a syngeneic tumor model. Our data are in line with the tumor suppressing activity of maspin as demonstrated in breast cancer model. Several recent clinical and experimental studies also support our conclusion. For instance, maspin gene expression is down regulated in prostate cancer cells (18) and also in clinical prostate cancer specimen (12). Recombinant maspin *in vitro* inhibits migration of prostatic cells (8). In a xenograft model. maspin was found to inhibit prostate osteolytic lesion and angiogenesis (19).

The molecular mechanism underlying maspin's tumor inhibition is still not fully understood. Clearly, regulation of angiogenesis is one mechanism by which maspin exerts its inhibitory role. Our study confirmed that C2N-46 maspin expressing tumor cells were inhibited in tumor forming ability, partially because of the inhibition of angiogenesis by the secretion of maspin protein. Another new finding from this study is that maspin expressing tumor cells in a 3D culture system underwent apoptosis in a significant higher rate than their C2N prostate tumor control. Under normal cell culture conditions in vitro, tumor cells are grown in the presence of an excess amount of serum and growth factors and therefore are less likely to undergo apoptosis. This is why the phenomenon of increased apoptosis has escaped people's attention in the past. Since maspin expressing tumors were completely inhibited in tumor formation assay, we examined the role of maspin in apoptosis in 3D tumor spheroids. The 3D model has a distinct advantage for both tumor-tissue interaction and for the interaction between normal epithelial cells and the extracellular matrix that surrounds them in forming life and death decisions (20). In the three-dimensional spheroid model, tumor cells in the center of spheroids have limited access

to growth factors, which mimics more closely solid tumors in vivo. Our data showed that the tumor spheroids formed from maspin-expressing tumors had a higher apoptosis rate than that from C2N tumor controls, indicating maspin is actively involved in the induction of prostate tumor cell apoptosis. Our data strongly suggests that the induction of apoptosis and inhibition of angiogenesis represent two major mechanisms by which maspin inhibits prostate tumor progression in vivo.

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Reference

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1998. CA Cancer J Clin, *48:* 6-29, 1998.

2. Navone, N. M., Labate, M. E., Troncoso, P., Pisters, L. L., Conti, C. J., von Eschenbach, A. C., and Logothetis, C. J. p53 mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. J Urol, *161:* 304-308, 1999.

3. Greenberg, N. M., DeMayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O., Cunha, G. R., Donjacour, A. A., Matusik, R. J., and Rosen, J. M. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A, *92:* 3439-3443, 1995.

4. Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C., and Greenberg, N. M. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res, *57:* 3325-3330., 1997.

5. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells [see comments]. Science, *263:* 526-529, 1994.

6. Zhang, M., Maass, N., Magit, D., and Sager, R. Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. Cell Growth Differ, *8:* 179-186, 1997.

7. Seftor, R. E., Seftor, E. A., Sheng, S., Pemberton, P. A., Sager, R., and Hendrix, M. J. maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res, *58:* 5681-5685, 1998.

8. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J., and Sager, R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci U S A, *93:* 11669-11674, 1996.

9. Zhang, M., Volpert, O., Shi, Y. H., and Bouck, N. Maspin is an angiogenesis inhibitor. Nat Med, *6:* 196-199, 2000.

10. Xia, W., Lau, Y. K., Hu, M. C., Li, L., Johnston, D. A., Sheng, S., El-Naggar, A., and Hung, M. C. High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. Oncogene, *19:* 2398-2403, 2000.

11. Chen, Z., Fan, Z., McNeal, J. E., Nolley, R., Caldwell, M. C., Mahadevappa, M., Zhang, Z., Warrington, J. A., and Stamey, T. A. Hepsin and maspin are inversely expressed in laser capture microdissectioned prostate cancer. J Urol, *169:* 1316-1319, 2003.

12. Machtens, S., Serth, J., Bokemeyer, C., Bathke, W., Minssen, A., Kollmannsberger, C., Hartmann, J., Knuchel, R., Kondo, M., Jonas, U., and Kuczyk, M. Expression of the p53

and Maspin protein in primary prostate cancer: correlation with clinical features. Int J Cancer, *95:* 337-342., 2001.

13. Abraham, S., Zhang, W., Greenberg, N., and Zhang, M. Maspin functions as tumor suppressor by increasing cell adhesion to extracellular matrix in prostate tumor cells. J Urol, *169:* 1157-1161, 2003.

14. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells [published erratum appears in Cell 1998 Jul 24;94(2):following 271]. Cell, *93:* 705-716, 1998.

15. Sutherland, R. M., McCredie, J. A., and Inch, W. R. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst, *46:* 113-120, 1971.

16. Zhang, M., Shi, Y., Magit, D., Furth, P. A., and Sager, R. Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene, *19:* 6053-6058., 2000.

17. Shi, H. Y., Zhang, W., Liang, R., Abraham, S., Kittrell, F. S., Medina, D., and Zhang, M. Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res, *61:* 6945-6951., 2001.

18. Zhang, M., Magit, D., and Sager, R. Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci U S A, *94:* 5673-5678, 1997.

19. Cher, M. L., Biliran, H. R., Jr., Bhagat, S., Meng, Y., Che, M., Lockett, J., Abrams, J., Fridman, R., Zachareas, M., and Sheng, S. Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. Proc Natl Acad Sci U S A, *100:* 7847-7852, 2003.

20. Jacks, T. and Weinberg, R. A. Taking the study of cancer cell survival to a new dimension. Cell, *111:* 923-925, 2002.

Figure Legend

Fig.1. Analysis of C2N prostate primary tumor, tumor vessel density, and lung metastasis by histology and immunohistochemistry. a. Histology of C2N tumor by H&E. Note tumor cells invade to the skin keratinocytes (arrow). B. Lack of tumor encapsulation in C2N tumor section. c. Immunohistolochemistry of CD31. Arrows indicate the tumor vessels. d. Histology of lung metastasis stained by H&E. Arrows indicate C2N tumor cells.

Fig. 2. C2N prostate tumor cells form 3D spheroids. a. Photograph of C2N tumor spheroid under inverted microscope. b. The C2N tumor spheroids were embedded and sectioned into 5 μM section. The section was stained by H&E.

Fig.3 TUNEL analysis of C2N and C2N-46 tumor spheroids. TUNEL staining of apoptotic cells in C2N (a) and C2N-46 (b) spheroids. Green cells indicated TUNEL positive cells. All cells were stained by DAPI (blue). c. Summary of TUNEL analysis for C2N and C2N-46 spheroids. Total number of cells counted were indicated as the N.

Fig.4. Secretion of maspin by C2N-46 and inhibition of endothelial cell migration. a. Western blot analysis of maspin using condition medium collected from C2N and C2N-46. Bacterial recombinant maspin protein (rMas) was used as control. Condition medium was concentrated 20X fold. b. The effect of maspin on HUVEC endothelial cell migration. HUVEC cells were treated by condition medium from C2N, C2N-46, C2N-46 plus preserum, C2N-46 plus maspin

antibody. Note condition medium of C2N-46 inhibited HUVEC cell migration and this effect was maspin specific. ** indicates statistical significance compared to C2N control ($p < 0.003$).

Table 1. Effect of maspin overexpression on prostate tumor development

		C2N-46	C2N	
Group 1,2	% of tumor development	0 % (0 /25)	90.5% (19/21)	P<0.001
	Tumor free period (days)	180	54.61±10.92	
	Tumor observation (days)		57.06±12.34	
	% of lung metastasis		42.1% (8/19)	

Figure 1

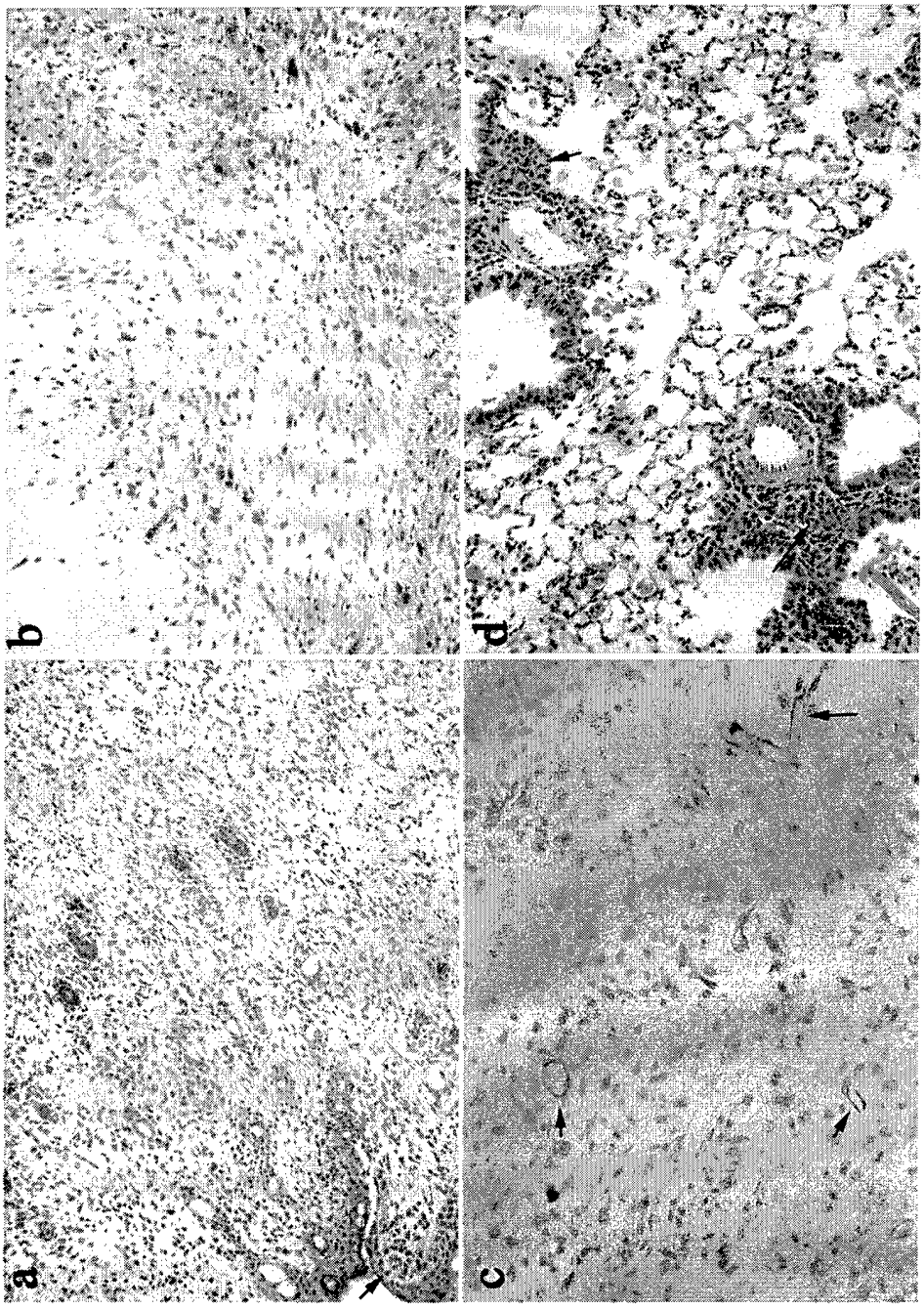


Figure 2

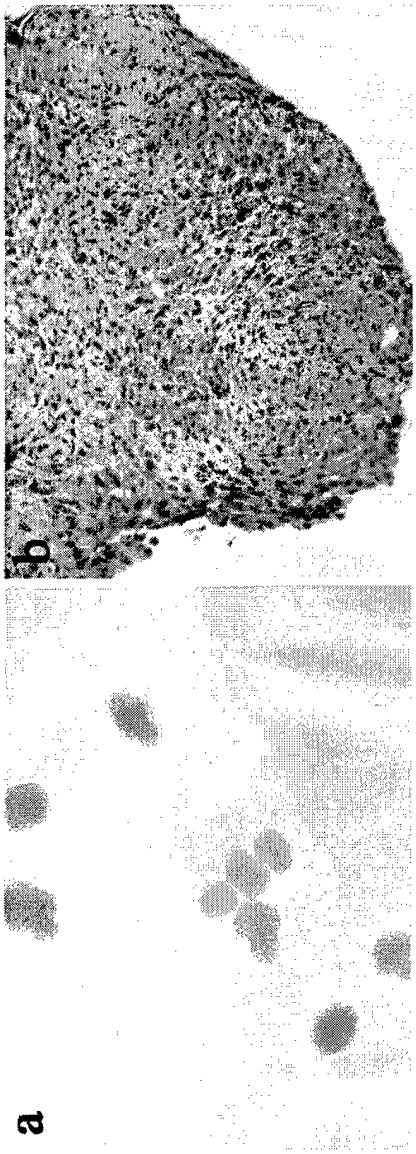
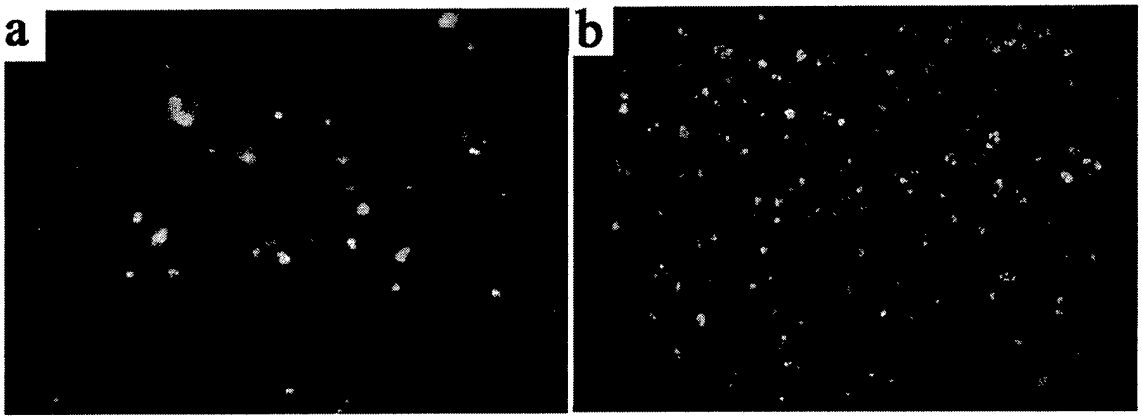


Figure 3


a
b

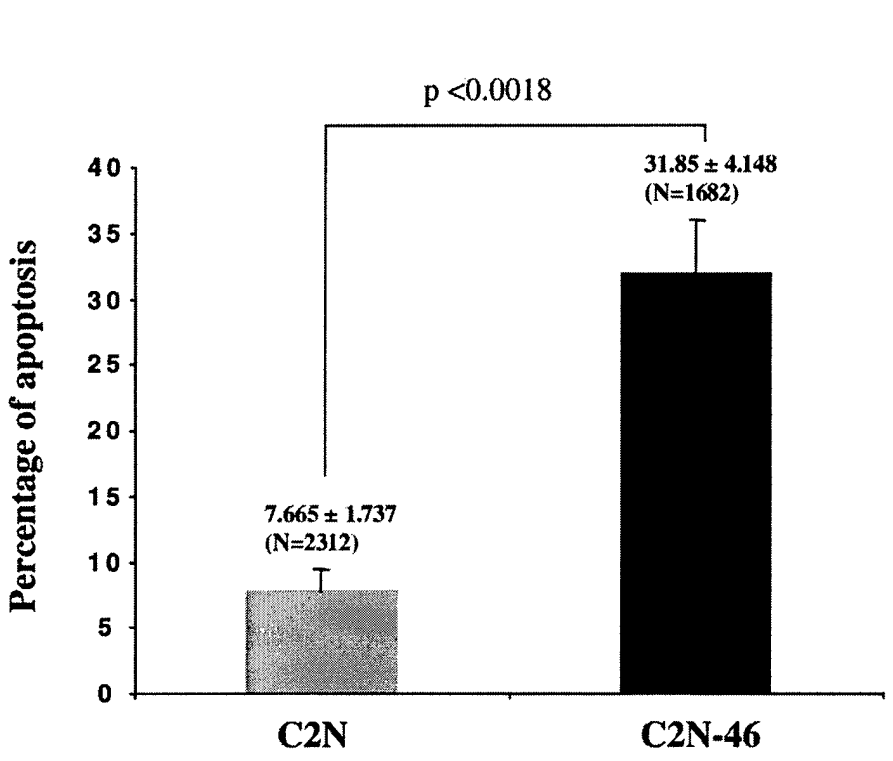
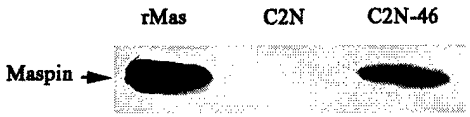
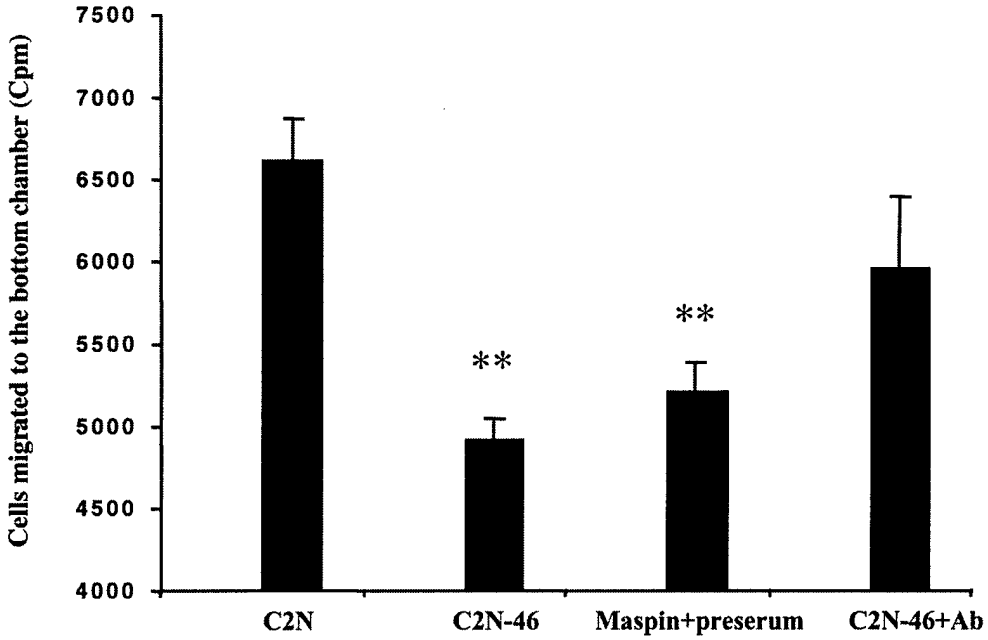

c
p <0.0018
31.85 ± 4.148
(N=1682)
7.665 ± 1.737
(N=2312)
Percentage of apoptosis
40
35
30
25
20
15
10
5
0
C2N
C2N-46

Figure 4

A


rMas
C2N
C2N-46
Maspin

B


Cells migrated to the bottom chamber (Cpm)
7500
7000
6500
6000
5500
5000
4500
4000
**
**
C2N
C2N-46
Maspin+preserum
C2N-46+Ab