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Genes involved in the control of tumor progression and their possible use for gene therapy

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Summary

Three major groups of genes may be used for cancer gene therapy: (*i*) oncogenes and tumor suppressor genes; (*ii*) genes involved in the control of tumor progression and metastasis; and (*iii*) genes encoding proteins protecting the organism from tumor cells. Each group contains numerous genes, and the discovery of new important genes is an exciting prospect in cancer research. We are working on the search and characterization of the genes over- or under-expressed in metastatic comparing to non-metastatic tumors of the same origin. Two mouse systems are being used: (*i*) VMR-0 (non-metastatic mammary adenocarcinoma cells) - VMR-100-Liv and VMR-100-Ov cells (metastatic preferentially to the liver or ovaries, respectively); and (*ii*) CSML-0 - CSML-100 (mammary adenocarcinoma cells non-metastatic and metastatic to the lungs, respectively). Several different genes were found to be over-expressed in metastatic cells, but only few of them were shown to be necessary and sufficient for maintaining the metastatic phenotype using stably transfected cells and/or transgenic animals. Among them are the *mts1* and *c-met* genes. The *mts1* gene, encoding a calcium-binding protein of 101 amino acids of the S-100 family, was extensively characterized. Its expression induced a number of changes in cell functions connected with cytoskeleton features, attachment properties of the cell, mesenchyme formation and possibly tumor vascularization. As a multifunctional regulator, the *mts1* gene is a promising target for gene therapy of cancer.

Other genes identified are over-expressed only in few metastatic tumors and do not seem to be connected directly with the acquisition of the metastatic phenotype. However, during the transfection experiments some interesting features emerged for these genes, raising the possibility of their exploitation in cancer gene therapy. The most interesting is the *tag7* gene encoding a new cytokine, 182 amino acids long, with a far distant relation to cytokines of the TNF-Lymphotoxin family. The *tag7* gene is expressed in lymphoid cells, in a limited set of other normal cells, and in few cancer cells including myelomas. The Tag7 protein is secreted to the culture medium and possesses a strong cytotoxic activity inducing apoptosis. VMR-0 cells were stably transfected with a construct containing the *tag7* gene under control of the CMV promoter. The original VMR-0 tumors killed mice in one month after subcutaneous transplantation; animals displayed large necrotic foci at this stage. However, the VMR-0/*tag7* cells, synthesizing very low amounts of Tag7 protein, exhibited dramatically different growth properties: they grew much slower; even after 4 months, no mice were killed by tumors arising from the transplanted cells and no necrotic foci were formed. Histological analysis of VMR-0/*tag7* tumors showed a strong inhibition in mitotic rates and an enhanced rate of apoptosis compared to VMR-0 tumors. The tumors induced by transplantation of a mixture of VMR-0 and VMR-0/*tag7* cells also grew much slower than VMR-0 cells alone, suggesting an activation of the immune system against tumor (tumor vaccination effect), which may be mediated through induction of CTL cells. Experiments with nude mice gave similar results. In fact at later stages of development in nude mice, VMR-0/*tag7* tumors were completely eradicated. It seems that the effect of *tag7* expression is complex and includes

activation of an immune response as well as a direct cytotoxicity. The higher *tag7* expression in culture cells is incompatible with cell survival. Experiments are in progress for further elucidating the role of Tag7 and its exploitation for the development of tumor vaccines.

I. Introduction

Three major groups of genes may be used for cancer gene therapy: (*i*) oncogenes and tumor suppressor genes; (*ii*) genes involved in the control of tumor progression and metastasis; and (*iii*) genes encoding proteins protecting the organism from tumor cells. Each group contains numerous genes, and the discovery of new important genes is an exciting prospect in cancer research. We are working on the search and characterization of the genes over- or under-expressed in metastatic comparing to non-metastatic tumors of the same origin. Below, we briefly summarize the general data on genes and proteins involved in the control of tumor progression (for more information see review articles in the Reference list). Thereafter, we present the data obtained in our laboratories on two genes from the second and third groups mentioned above. At least one of these genes and its protein product may be used for the gene therapy of cancer.

II. Tumor progression and tumor metastasis

The tumor is not a static formation. It is developing and changing constantly. This depends on the genetic instability of the tumor. As a result of transformation, tumor cells acquire a partial independence from regulatory signals arising from neighboring cells and grow more or less independently of these signals. Another important feature is the elimination of the cells with damaged DNA. Normally such cells cannot overcome the cell cycle checkpoints and progress through the apoptotic process leading to their death. The p53 protein plays an important role in the direction of damaged cells along the apoptotic way. Many mutations in the *p53* gene, frequently occurring in tumors, lead to the loss in the ability to induce apoptosis and to down regulate cell proliferation. As a result, cells become able to survive after DNA damage, and this results in the increase in mutation rate in such cell populations. Interestingly, another protein involved in the control of apoptosis, the product of the *bcl-2* gene which down regulates apoptosis, is a potential oncogene.

This, and possibly some other processes, result in accumulation of different types of mutations in tumor cells: translocations, loss of heterozygosity, point mutations and transpositions. Consequently, this leads to an accumulation of heterogeneity in tumor cell populations. If the mutated cell acquires some advantage for rapid growth or other properties useful for the cell itself, it has some good chances for survival and multiplication. Such changed cells may replace the original population of tumor cells over time. This phenomenon is known as tumor progression, that usually leads to appearance of a more malignant phenotype. Usually the same tumor contains cells with different genotypes and several clones obtained from the same tumor may differ in the level of malignancy.

It should be pointed out that in some cases, the malignant phenotype can appear just at the first stage of tumor development, as for example, in mouse mammary tumors induced by activation of the *neu* oncogene. However, in many cases, the malignancy develops in the course of tumor progression. One of the major features of malignant tumor cells is the ability to give metastases, i.e. new foci of tumor growth in distantly located regions of the organism.

The process of tumor progression and tumor metastasis is very complex and includes several independent steps: 1) vascularization of a primary tumor node; 2) detachment of tumor cell from the primary focus; 3) invasion into surrounding tissues including the blood vessels; 4) transfer to the new site and arrest at this site; 5) adhesion to the endothelial cells; 6) extravasation; 7) vascularization of a novel focus and its invasive growth at the new place. Each step depends on new special properties of tumor cells, such as ability to induce angiogenesis, detachment from or attachment to cell aggregates, cell invasiveness and cell motility (**Fig. 1**).

Each step is a complex one and is controlled by a number of genes and proteins. Therefore, one can expect a number of genes/proteins to be involved in the control of tumor metastasis. Several activated oncogenes themselves can generate a metastatic phenotype. However, in many other cases, the processes of oncogenesis and metastasis are uncoupled, and special genes are responsible for the appearance of the metastatic phenotype.

The genes for tumor progression and metastasis may be separated into two groups: effector genes and upstream regulatory genes. The protein products of effector genes directly determine the invasiveness and other features of the metastatic tumor, while proteins encoded by the genes in the second group act in an indirect way. They either control the expression of different effector genes or control some general cell function indirectly determining the features characteristic of malignant tumor cell.

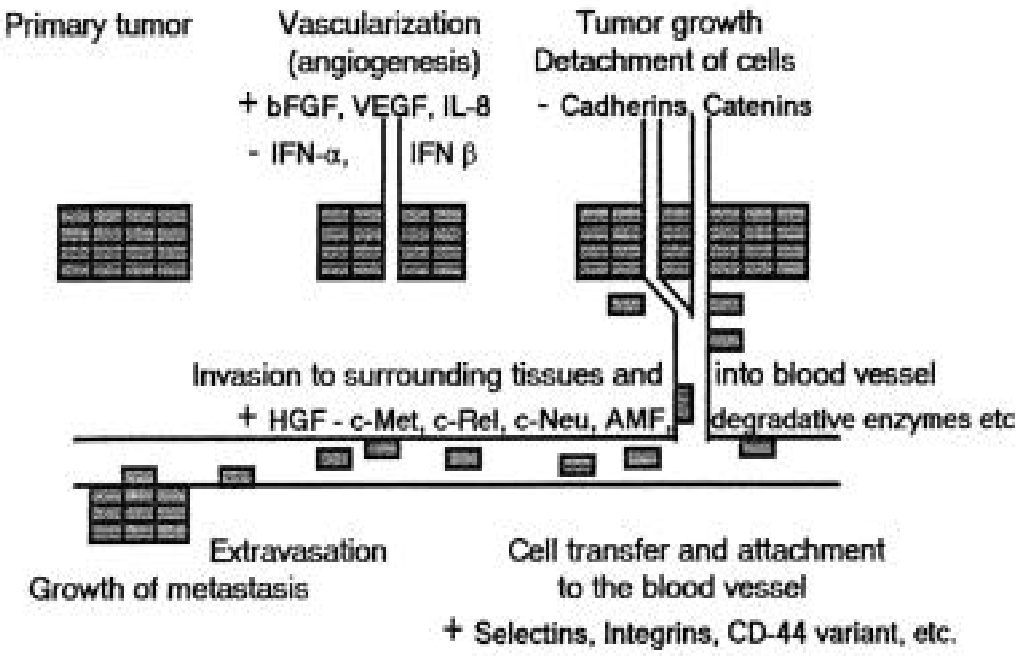

Fig. 1. The schematic presentation of tumor progression and metastasis.

Some proteins activating (+) or suppressing (-) tumor progression are indicated.

III. Some main steps of tumor progression and some genes involved in their control

A. Angiogenesis.

Before vascularization, or angiogenesis, takes place the tumor can only grow up to 2 mm in diameter due to the shortage of nutrition. After vascularization has occurred, tumor cells can grow to much larger dimensions. In addition, tumor cells from vascularized tumors can penetrate into the blood vessels and this is the first step for metastasis development.

The invasion of endothelial cells into the tumor node and formation of capillary sprout are influenced by many different cytokines produced either by tumor cells or by normal inflammatory cells whenever inflammation accompanies tumor development. There are positive and negative cytokines. Among positive cytokines inducing tumor angiogenesis are fibroblast growth factors, especially bFGF, vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and many others. On the contrary, cytokines such as interferons (IFN-α and IFN-β), angiostatic cartilage-derived inhibitors and several others inhibit angiogenesis in tumor foci. The production of corresponding cytokines by tumor cells depends on the complex interactions between tumor and surrounding host cells. Sometimes, angiostatic cytokines are produced by primary tumor itself and this prevents overgrowth of metastases. In such cases, surgical removal of the primary focus may lead to induction of metastasis. The control of angiogenesis by antiangiogenic polypeptides, like angiostatin, endostatin and some other agents is an important approach for cancer therapy (see this volume).

B. Detachment of tumor cells from the original cell cluster.

The next important step in metastasis is the detachment of tumor cells from aggregates to enter the blood vessel. Cell aggregation depends on several factors, the most important being represented by cadherins, immunoglobulins, integrins and selectins.

Aggregation of the cells plays an especially important role in the progression of the cancers, or epithelial tumors, as epithelial cells originally form firm aggregates and have very little motility. They bind to each other and to the basal membrane. Adherence junctions between normal and tumor epithelial cells depend on the cadherin family of proteins. The best characterized member of the family, E-cadherin, is a ca. 120 kDa protein with a large N-terminal extracellular part containing four calcium-binding domains, a transmembrane domain and a C-terminal intracellular domain. The extracellular domains of E-cadherins located on different cells interact in a calcium-dependent way. The C-terminal domain interacts with the C-terminal region of β-catenin (plakoglobin), whose N-terminal part binds to α-

catenin which interacts with the cytoskeleton. This chain of protein-protein interactions firmly holds epithelial cells at their position.

There is a strong correlation between cell behavior and E-cadherin content (Vlamincks *et al.* 1991). In transfection experiments, stable transfection and expression of the *E-cadherin* gene strongly suppresses the metastatic phenotype of different tumor cell lines. On the other hand, antibodies to E-cadherin make the tumor cells more aggressive in some cases. Mutations in the *E-cadherin* and β-*catenin* genes leading to their inactivation have been observed in some tumors and found to be associated with enhancement of metastatic phenotype. Down regulation of *E-cadherin* gene expression leads to a similar effect. Detachment from the cell aggregate leads to the aquisition by epithelial cells of several properties typical of mesenchymal cells: mesenchymal transformation of epithelial cells takes place. This phenomenon correlates neatly with the increase of invasiveness and appearance of metastatic phenotype in tumor cells (see also below).

C. Invasive growth.

Different groups of genes/proteins are involved in determination of invasive tumor growth. Examples include the tyrosine protein kinase-type receptors, the autocrine motility factor (AMF) and its receptor and different types of degradative enzymes.

Among the genes encoding tyrosine kinase receptors, *c-met, c-neu, c-ret* and *c-ros* were shown to be associated with the invasiveness of tumor cells. In more detail, the pair consisting of the ligand, scattering factor (SF)/hepatocyte growth factor (HGF), and of the receptor, c-Met, was studied. Their interaction leads to the induction of liver morphogenesis and at the same time to the increase of motility and invasiveness of tumor cells into the collagen matrix. The synthesis of both c-Met and SF/HGF is increased in different malignant tumors, in particular, in many cases of metastatic human breast cancer. In contrast to effector proteins, the system SF/HGF-cMet is part of the control proteins determining signal transduction resulting in the change of expression of several different genes.

AMF is a 64 kD protein that interacts with the receptor, 78 kD glycoprotein, gp78, and activates cell motility. The synthesis of the components of this ligand-receptor system is activated in parallel with progression of some tumors, for example, bladder carcinoma.

A wealth of data have been obtained on the role of different degradative enzymes and factors in tumor progression. Examples of degradative enzymes include different metalloproteinases and serine proteases, such as cathepsins, collagenases, stromelysins; plasminogen activator and its inhibitor, as well as gualuronidase and several other enzymes destroying extracellular matrix. The genes encoding these degradative enzymes are frequently activated in metastatic and, in general, in invasive cancer cells, although the correlation is not absolute. This group of enzymes may obviously play a role in degradation of extracellular matrix synergizing to the overgrowth and invasion of cancer cells, and in particular their invasion into blood vessels.

D. Attachment to endothelial cells (arrest in capillary bed) and extravasation.

The process of attachment of tumor cells to endothelial cells is induced by cytokines produced in tumor or inflammatory cells. These are IL-1, TNF, lipopolysaccharides (LPS) etc. Weak attachment is mediated by selectins. Synthesis of E-selectin is induced in endothelial cells. Its extracellular part interacts with tumor cell or leukocyte carbohydrates through an N terminal lectin-like domain. Strong attachment is mediated by interaction of integrins of tumor cells with the members of immunoglobulin superfamily located on the surface of endothelial cells. For example, α4β1 integrin usually present in melanomas and sarcomas binds to VCAM-1 (vascular cell attachment molecule), while α6β1 (present in colon carcinomas) and α6β4 (present in lung carcinomas) bind to ICAM-1 (intercellular cell attachment molecule). These interactions are responsible for a firm attachment.

Several reports have appeared indicating the special role in tumor metastasis of the CD-44 transmembrane hyaluronate receptor. Both metastatic and non-metastatic tumors contained the major variant of this protein, but only metastatic cells contained some minor variants of the protein characterized by the presence of additional domains, that were found to be responsible for intercellular interactions. The appearance of such variants was a result of alternative splicing that led to inclusion into mRNA of additional small exon(s). Stable transfection of non-metastatic cells with the construct expressing the CD-44 variant in some cases led to the enhancement of metastatic potential, although some opposite results were also obtained. Since both the attachment and detachment of cells play a role in metastasis just at different stages of the process, these controversial results may not be too surprising. Further experiments are needed before final conclusions can be reached.

Many other genes/proteins important for tumor progression have been described; the examples mentioned here are those of extensively studied. Some additional genes will be mentioned in further discussion.

Table 1. The genes with changed expression detected in the VMR-0 - VMR-100 and CSML-0 - CSML-100 pairs of tumor cells and/or tumors

Cell-line system / The gene or protein	Metastatic tumors comparing to non-metastatic
VMR-100 / VMR-0	
*tag7**	selectively expressed
c-met	over-expressed
novel serine threonine protein kinase*	selectively expressed
LAR (tyrosine protein phosphatase)	over-expressed
MHC class I H2-L-a antigen	over-expressed
Na,K-ATPase, catalytic sub-unit	over-expressed
Prothymosin	under-expressed
Cathepsin D	over-expressed
MMTV LTR	over-expressed
CSML-100 / CSML-0	
*mts1**	selectively expressed
ly6	selectively expressed
new Semaphorin*	selectively expressed

*Novel, previously undetected genes.

IV. Search for new genes involved in the control of tumor metastasis and the systems used.

Each of above mentioned genes plays a certain role in the acquirement by tumor cells of an invasive and metastatic phenotype. Probably, in different cases, different genes may be involved; one could expect that a number of genes controlling metastasis are still unknown. In particular, this may include upstream genes that are not directly involved in cell functioning but control other genes or the activity of proteins. An important direction for further studies is to discover such genes and to understand the role of their protein products in tumor progression and metastasis. In this respect, the most interesting are genes that either play a key role in producing a metastatic phenotype in various tumors or which can potentially be exploited for metastasis diagnostics and/or treatment.

Different approaches can be used in the search of new genes involved in the control of tumor progression. One approach is to search for genes, whose protein products are directly connected with tumorigenesis and metastasis. In this case, one can usually expect to find the "effector genes" encoding proteins directly participating in corresponding cell functions.

Another approach is a less direct, but still may give interesting results. It is the search for genes over-expressed or under-expressed in metastatic tumor cells compared to non-metastatic tumor cells of the same origin. In an ideal case, one can discover a gene belonging to a class of upstream genes with wide functions in the generation and maintenance of the metastatic phenotype. In many other cases, some "occasional" genes may be fished out, that are differentially expressed only in few types of tumor cells. Yet, in many cases, these new genes may become valuable for understanding several aspects of tumor progression and, more important, in development of the methods for gene therapy. For example, one such gene identified from our experiments, *tag7*, seems to belong to the group of genes encoding proteins protecting the organism from tumor cells, and its transfer to cancer patient could constitute an approach for gene therapy. Another gene discovered in our laboratory, *mts1*, may play an important role in metastasis as an upstream regulator gene.

Two mouse systems elaborated in the Russian Oncology Center (E. Revasova and V. Senin) have been used in our experiments. Both use the cell lines obtained from spontaneous mammary adenocarcinomas. The first is represented by a pair of VMR-0 non-metastatic cells and VMR-100 metastatic cells. Originally, VMR-0 cells were obtained and maintained as a cell line by subcutaneous transplantation. Occasionally, metastatic foci appeared, and the cells from these foci were taken for preparing cell culture. As a result of such selection, highly metastatic cell lines were obtained with preferential metastasis to the liver (VMR-100-Liv cells) or to the ovaries (VMR-100-Ov cells). Another pair are the CSML-0 and CSML-100 cells, non-metastatic and highly metastatic to the lungs, respectively, obtained in about the same way, also from spontaneous mammary adenocarcinoma. These two pairs of cell lines were further used for screening of the genes differentially expressed in metastatic cells. Two technologies were used: subtraction of cDNA libraries at the earlier stage and the mRNA display method at the later stage of the screening methodology. The mRNA display method, although giving a lot of false clones, is still much easier to apply and, ultimately, more genes of interest may be obtained with this technique.

Several different genes were found to be over-expressed or under-expressed in metastatic cells in the two pairs

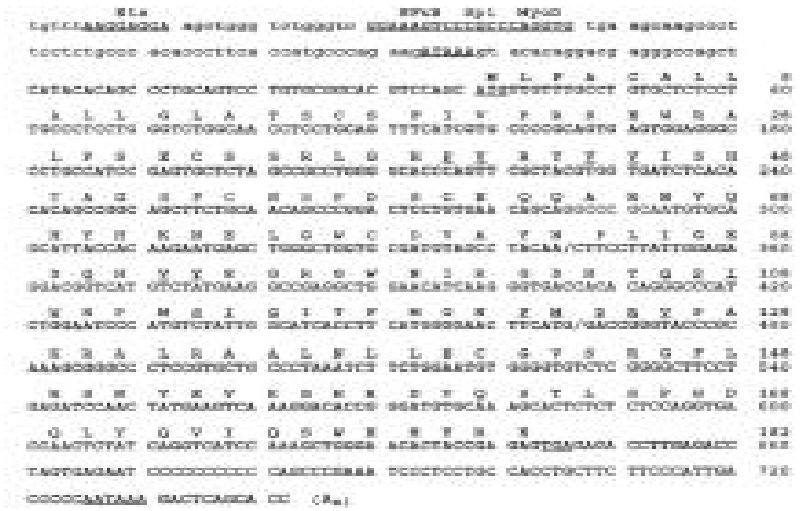

Fig. 2. Nucleotide sequence of the *tag7* gene (exons and upstream sequences) and amino acid sequence of Tag7 protein.

The transcription factor-binding sites, promoter, initiation and termination codons and AATAAA signal are underlined. The symbol / means borders between exons. In the amino acid sequence, the homologies to the TNF-Lymphotoxin family are underlined.

described above (**Table 1**). One of them, the *mts1* gene was found to be over-expressed in many different metastatic tumors and also shown to be necessary and sufficient for maintaining the metastatic phenotype at least in some tumor cells in experiments on stably transfected cells and on transgenic animals. Several other new genes may arise; however, additional experiments on stable transfection of cells with the corresponding gene constructs followed by phenotypic analysis, are required.

Some of the discovered genes correspond to those described before, and are mostly effector genes (see **Table 1**). Among the upstream genes, the *c-met* gene seems to be an important regulator of tumor cell invasiveness and, as a result, of metastatic behaviour. It is the receptor for the SF/HGF (see above). Interestingly, the difference in expression of *c-met* between VMR-100 and VMR-0 cells appeared only *in vivo*. The effect can be mimicked in cell culture after contact with stromal cells. The *c-met* gene expression is used as a test for the level of malignancy.

Finally, some of the discovered genes were over-expressed only in few types of metastatic cells, and therefore, they could not play a general role in determining the metastatic phenotype. This was confirmed in transfection experiments. Among them are the gene encoding a novel serine-threonine protein kinase and the *tag7* gene, both over-expressed in VMR-100 cells. Although, transfection with the *tag7* construct did not induce a metastatic phenotype, it strongly influenced the properties of tumor cells. Therefore, this gene and its protein product were studied in more detail.

V. A novel cytokine, Tag7, and its properties

A. Structure of the *tag7* gene.

The *tag7* gene was obtained by the mRNA display method, as a gene over-expressed in VMR-100-Liv and VMR-100-Ov tumors growing in mice after subcutaneous transplantation, as compared to tumors induced by VMR-0 cells (Kustikova *et al.* 1996). The full-length cDNA clone as well as a genomic clone were obtained and sequenced. The putative Tag7 protein consists of 182 aminoacids with no homology in the data-base (**Fig. 2**). Three exons were determined. The upstream region of the *tag7* gene contains several binding sites for well known transcription factors, Ets1, NFκB, Sp-1 and MyoD. It was noticed that the arrangement of these sites was similar to that in the gene encoding Lymphotoxin-β. A careful manual comparison of the Tag7 and TNF-Lymphotoxin amino acid sequences revealed a very low level of homology in different regions (**Fig. 2**), suggesting Tag7 to be a far distant relative of the members of TNF-Lymphotoxin family of cytokines.

The *tag7* gene is located on the seventh mouse chromosome in the A3 region, i.e. in a different chromosome than the *TNF* gene cluster. The 7A3 chromosomal region is interesting, as it has a genetic relation to the development of such autoimmune diseases, as *lupus erythematosus.*

B. Expression of the *tag7* gene in normal tissues and tumors.

High expression of the *tag7* gene is not a general characteristic feature of metastatic tumors (**Fig. 3**). For example, CSML-100 cells do not express the *tag7* gene, whereas CSML-0 cells do. Most metastatic and non-metastatic tumor cells tested do not express the gene at all. Thus, it is clearly not a marker for the metastatic pheno-

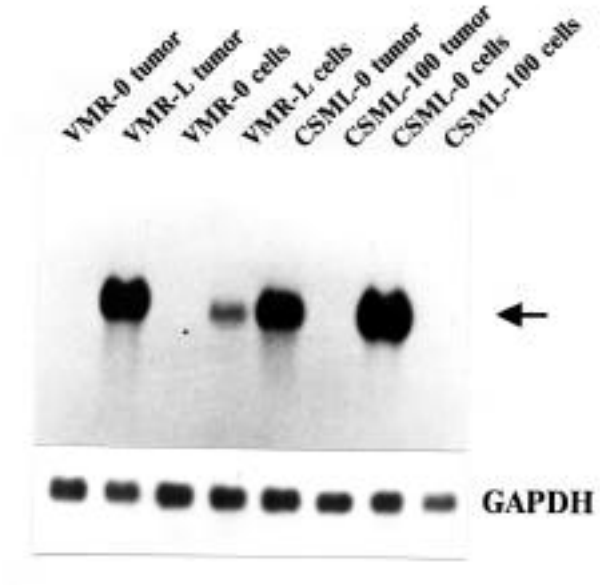

Fig. 3. Northern blot hybridization of RNAs from cell culture and tumors with the *tag7* probe type.

Exceptionally, the human multiple myeloma cells all express the *tag7* gene at a rather high level. Strong *tag7* expression in VMR-100 cells takes place only in tumors *in vivo*. In cells in culture, the level of *tag7* mRNA is very low, suggesting that active *tag7* expression depends on interactions between tumor and host (possibly stromal) cells.

Expression of the *tag7* gene takes place in several tissues of normal organism (**Fig. 4**). The highest signal after Northern blot hybridization was obtained in the spleen and lungs. It is actively expressed in isolated lymphoid cells, circulating monocytes, thymocytes, splenocytes and resident peritoneal macrophages. The level of *tag7* mRNA synthesis and Tag7 protein accumulation in cultured splenocytes is moderately enhanced by LPS induction (but not by IL-2 or PHA). The LPS effect on TNF and Lymphotoxin-a expression is much stronger and faster. Interestingly, *in situ* hybridization reveals the active *tag7* expression in some specific cell sets in different organs, for example in Purkinje cells of cerebellum and in some neurons of hippocampus (**Fig. 4**). Its expression was found in the duodenal cells of 7-8 day embryos.

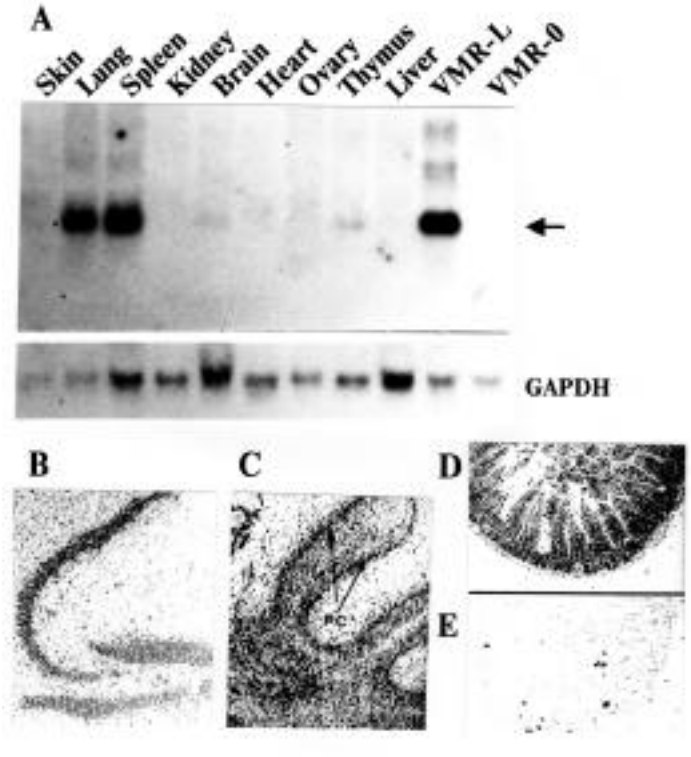

Fig. 4. Expression of the *tag7* gene in normal tissues.

A. Northern blot analysis of RNAs prepared from different mouse tissues.

B-E. *In situ* hybridization of adult mouse tissues with *tag7* cRNA probe. B, hippocampus; C, cerebellum Purkinje cells, PC, are intensively labeled; C, intestinal section; E, the same after RNase digestion.

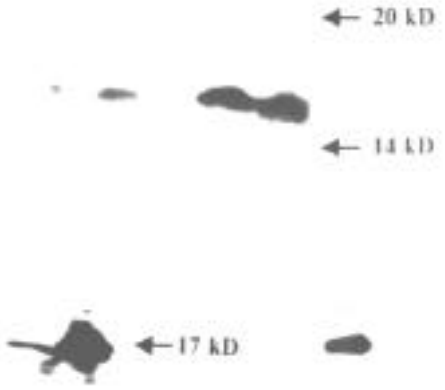

Fig. 5. Tag7 protein exists in soluble and cell-associated forms. Western blot analysis with affinity-purified antibodies to Tag7 protein. 1, Freshly isolated splenocytes; 2, 3, Splenocytes in culture after 0.5 h. LPS induction; 4, 5, The same after 24h induction; 6, 7, VMR-100-Liv cells after LPS stimulation. 1, 2, 4, 6, Tag7 protein from the cells; 3, 5, 7, Tag7 protein from the culture medium. 8, Recombinant Tag7 protein.

C. Properties of the Tag7 protein.

The recombinant Tag7 protein was obtained in inclusive bodies of *E. coli* and used for preparing polyclonal and monoclonal antibodies. Western blot analysis showed the presence of Tag7 protein both in the cells and in the culture medium (the major part) of Tag7-producing cells (**Fig. 5**). Thus, Tag7 is a secreted protein. Tag7 protein possesses a rather strong cytotoxic activity in respect to several cell lines, in particular, mouse L929 and human Jurkatt and MCF-7 cells (**Fig. 6**). Affinity-purified polyclonal or monoclonal antibodies destroy the cytolytic activity of Tag7 protein, while antibodies to TNF and Lymphotoxin-β do not. *Vice versa*, antibodies to Tag7 are not efficient in preventing the cytotoxic effect of TNF. The cytotoxicity of Tag7 is higher than that of the best commercial TNF preparation at the same concentration. The cytotoxicity of Tag7 protein is mediated through apoptosis as deduced from cytological analysis and the appearance of oligonucleosomal DNA repeats in the nuclei of target cells (**Fig. 6**).

High level of cytotoxicity of Tag7 protein creates a number of experimental problems. Synthesis of recombinant Tag7 in the periplasm of bacterial cells kills them. In transfection experiments, the cells producing high amount of Tag7 rapidly die. Therefore, only a limited amount of Tag7 protein can be obtained from the conditioned medium from the cultured cells producing small amount of Tag7. The problem of obtaining of native Tag7 from inclusive bodies has not yet been solved.

VI. Possible exploitation of the *tag7* gene for antitumor gene therapy

A. Influence of *tag7* expression on the growth of VMR-0 tumors.

The possibility to use the *tag7* gene for cancer gene therapy was revealed from transfection experiments aimed to analyse the role of Tag7 in tumor metastasis. VMR-0

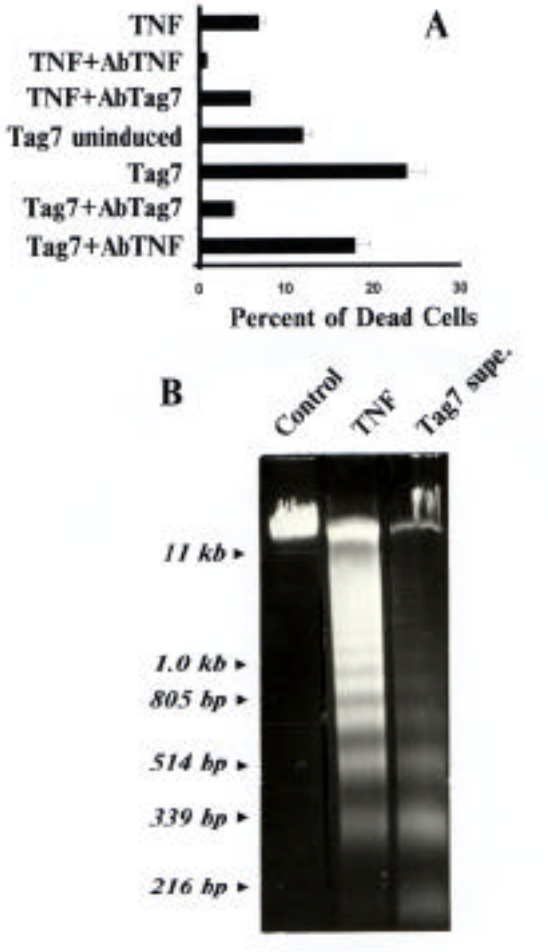

Fig. 6. Cytotoxicity of Tag7 protein. The L929 cells were incubated with Tag7 from VMR-100-Liv supernatant or with commercial TNF. In some experiments the indicated antibodies were added.

A. Cell death assayed by trypan blue staining; **B**. Appearance of nucleosome repeats after incubation of cells with TNF and Tag7.

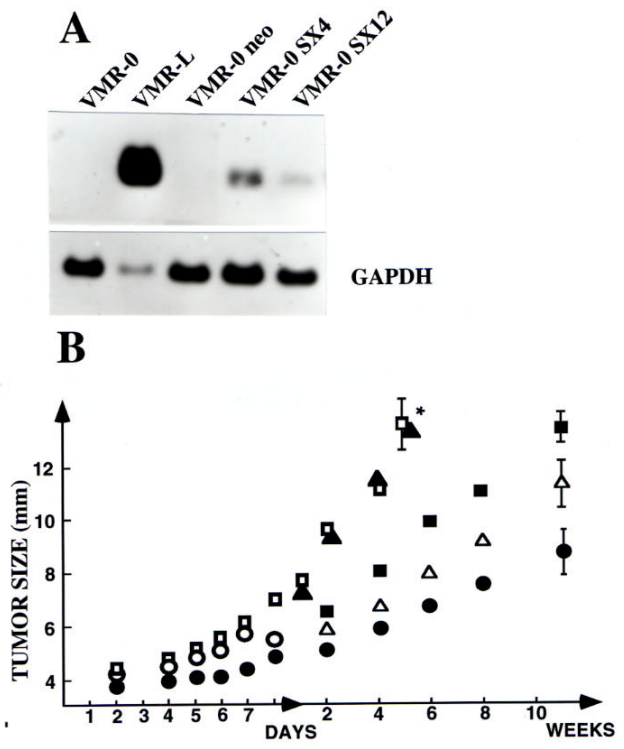

Fig. 7. Influence of *tag7* expression on the tumor cell growth.

A. Northern blot hybridization of RNAs from different VMR/*tag7* cell lines (SX4 and SX12) with *tag7* probe. The level of *tag7* expression in transfected cells is much lower than in VMR-100-Liv.

B. 10^6 VMR-0 cells (□), mock-transfected VMR-0/Neo (▲) and *tag7* transfected SX4 (●) and SX12 (Δ) cells were subcutaneously injected to 10, 5, 10 and 10 A/Sn mice, respectively. The mean values of tumor size were determined at different periods after injection. SX4 cells expressed *tag7* at higher level, than SX12 cells. (❍). Inhibition of SX4 effect by purified polyclonal antibodies to Tag7 (3 mice). (■), 10^6 VMR-0 cells were coinjected with 10^6 SX4 cells (3 mice). *Animals of these groups died 4-5 weeks after injection.

cells were stably transfected with a construct containing the *tag7* gene under control of the CMV promoter. Two stable cell lines, VMR-0/*tag7*, were obtained, both expressing *tag7* at a low level. Yet, the level of expression in one cell line was two- three-fold higher than in another. It should be pointed out that the growth rate of VMR-0/*tag7* cells in culture was the same as of control VMR-0 cells. However, the attempts to obtain the higher level of *tag7* expression in VMR-0 cells led to the inhibition of growth of the cells in culture followed by their death at later stages.

The VMR-0/*tag7* cells (10^6) were subcutaneously transplanted to isogeneic mice. The untreated VMR-0 cells or cells transfected with neomycin gene alone were used as a control. The original VMR-0 tumors grow fast at the site of injection and kill mice in one month after subcutaneous transplantation. The tumors contain large necrotic foci at this stage (**Fig. 7**).

The VMR-0/*tag7* cells have a dramatically changed growth properties. They grow much slower. Even after 4 months, no mice were killed by the tumor. No necrotic foci were formed, even at later stages, when the tumors reached a large size. Histological analysis of VMR-0/*tag7* tumors recovered strong inhibition of mitotic rate and activation of apoptosis frequency comparing to VMR-0 tumors (**Table 2**). Growth inhibition was much stronger in the case of VMR-0/*tag7* cell line producing higher amount of Tag7. The injections of antibodies to Tag7 accelerated the tumor growth at the period of their application (**Fig. 7**).

The tumors induced by transplantation of the mixture of VMR-0 (10^6) and VMR-0/*tag7* (10^6) cells also grow much slower, than VMR-0 cells alone (**Fig. 7**), suggesting the activation of immune system against tumor cells (tumor vaccination effect), which may be realized through formation of CTL cells. This interpretation is supported by observation, that the growth of tumor cells of another line (CSML-100) is not inhibited by cotransplantation with VMR-0/*tag7* cells.

Table 2. Influence of the *tag7* expression on the growth properties of VMR-0 cells *in vivo* in isogenic mice.

Tumor cells	Mitotic cells	Apoptotic cells	Ratio M:A
(*i*) VMR-0	3-5%	<0.5%	ca. **10/1**
(*ii*) VMR-0/*tag7*	<1%	5-8%	ca. **1/10**
Ratio, (*ii*):(*i*)	ca. **1/5**	ca. **20/1**	ca. **1/100**

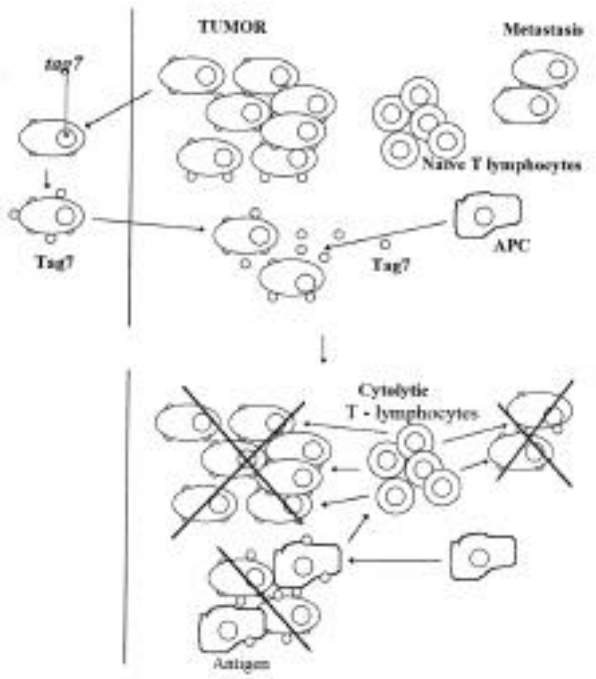

Fig. 8. The scheme with a possible explanation of the inhibition of tumor growth by activation of cytolytic T lymphocytes.

APC-antigen-presenting cells. Filled circles, Tag7; empty circles, tumor antigens.

B. On the mechanism of tumor growth inhibition by *tag7* expression.

It is usually accepted that tumor cells expressing some cytokines, in particular GM-CSF or IL-2, attract the antigen presenting cells (APC). Then, APC convert naive T-lymphocytes into cytolytic T-lymphocytes (CTL) recognizing the antigens present in tumor cells and attacking both primary foci and metastases. One can suggest, that VMR-0/*tag7* cells possess the same properties (**Fig. 8**).

To check this, the experiments with nude mice were performed as they lack or have a very weak system for T lymphocyte response. The results were very similar. VMR-0/*tag7* cells grow much slower, than VMR-0 cells. VMR-0 cells killed mice in 2 months, i.e. later than in the case of isogenic mice. However, the general properties of growth and tumor morphology remain unchanged (**Fig. 9**). The VMR-0/*tag7* cells grow in nude mice even slower. The tumors increased in size at the beginning but later on they frequently decreased in size. Some waves of growth followed by degeneration could be observed. Finally either

Fig. 9. Nude mice injected with 10^6 VMR-0 (**A**) or SX4 (**B**) cells after 2 months of tumor development.

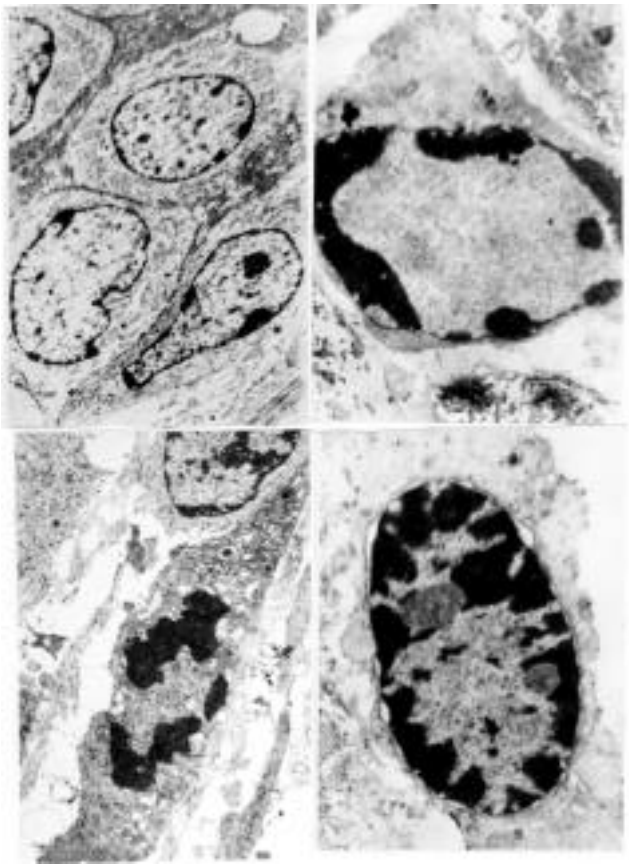

Fig. 10. EM pictures of mitotic (M) and normal (N) cells in VMR-0 tumor and apoptotic cells (A) in SX4 tumor transplanted to nude mice.

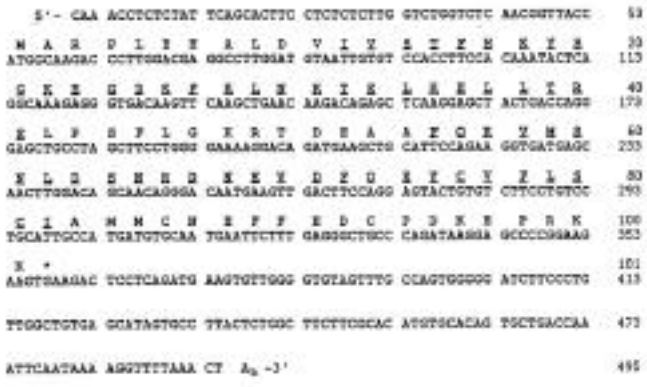

Fig. 11. Nucleotide sequence of the *mts1* gene coding sequence and amino acid sequence of Mts1 protein.

In the amino acid sequence, the calcium-binding domains are underlined.

small tumors remained or complete dissolving of the tumor took place (**Fig. 9**). Histological analysis again shows strong inhibition of mitotic rate and at least tenfold increase in frequency of apoptosis. Typical pictures of apoptosis are observed under electron microscopy (EM) (**Fig. 10**). It seems that the effect of tag7 expression is rather complex and at least includes activation of an immune response mediated by CTL cells and direct cytotoxicity of the Tag7 protein. Experiments are in progress for more precise understanding of Tag7 action and for its application to cancer treatment.

VII. The *mts1* gene

A. General properties of the *mts1* gene.

Another extensively studied gene is the *mts1* gene. It may play an important role in the control of tumorprogression and appearance of metastases at least in some tumors. The *mts1* gene has been discovered in experiments on cDNA libraries subtraction using CSML-100 and CSML-0 cell lines (see above). The *mts1* gene is transcribed to a 0.55 kb mRNA, which was abundant in CSML-100 cells and absent from CSML-0 cells. This mRNA was detected in many metastatic tumor cell lines of different origin, but not in non-metastatic tumors, although several exceptions could be observed. Therefore, the gene was called as *mts1*, a gene encoding Metastasin 1 protein, Mts1 (Ebralidze *et al.* 1989).

The cDNA was sequenced and the protein structure was deduced. Mts1 was found to be a protein 101 amino acid long with two typical calcium-binding domains (**Fig. 11**). It belongs to the S-100 sub-family of calcium-binding proteins. The *mts1* gene was described at about the same time in several other groups under different names, but without any relation to tumor metastasis. The *mts1* has also been cloned from the human genome. Human Mts1 protein differs from its mouse counterpart just by 7 amino acid substitutions.

The *mts1* gene is expressed in several normal tissues: embryonic fibroblasts, trophoblasts, and lymphoid cells, in particular, in T-lymphocytes and activated macrophages. At least some of these cells possess invasive properties. The level of *mts1* expression can be readily modulated by different lymphokines or calcium ionophores (Grigorian *et al.* 1993).

No sequence rearrangement usually takes place during the change of tumor phenotype to a metastatic one, as deduced from Southern blot hybridization experiments. The only exception was observed in the VEHI-3 cell line (myelomonocytic leukaemia), where a deleted copy of IAP retrovirus-like mobile element was found to be inserted into the first intron of the *mts1* gene. As a result, the

transcription was started within the IAP LTR, and chimeric mRNA was synthesized, while the protein remained unchanged (Tarabykina *et al.* 1996). Therefore, activation of *mts1* mRNA synthesis should usually result from changes in concentration of certain trans-regulatory factors. Finding of such factors responsible for *mts1* activation may lead to discovery of new genes involved in the creation of metastatic phenotype, acting upstream in respect to the *mts1* gene.

The *mts1* gene consists of three exons and two introns. The first exon is small and does not contain translated sequences. The gene is located in the gene cluster containing several other members encoding proteins belonging to S-100 family. The distances between genes in the cluster are rather small. Examination of the *mts1* upstream region up to the 3'-end of the neighbor gene of the cluster has led to the conclusion that cis-regulatory elements are not present but instead a TATA-box containing promoter. All cis-regulatory elements have been found within the first intron. Several well known positive and negative cis-regulatory element binding was determined as well as some new transcription factors have been found (**Fig. 12**) (Grigorian *et al.* 1993, Tulchinsky *et al.* 1996, 1997).

The first element (from the 5'-end of the intron) is the enhancer of a moderate strength possessing no homology

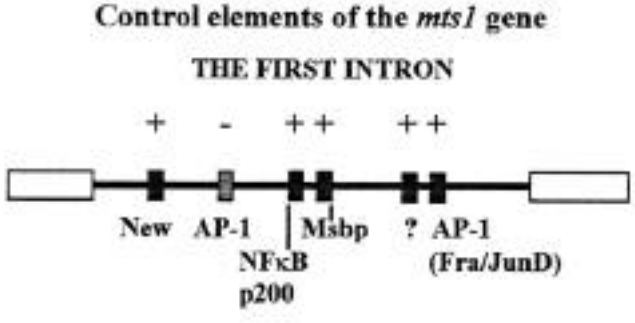

Fig. 12. Regulatory region of the *mts1* region located in the first intron.

with known enhancers. It binds a novel transcription factor, which is present in both CSML-100 and CSML-0 cells. However, *in vivo* the protein binding was detected only in CSML-100 cells. Thus, the enhancer selectively works in metastatic cells in spite of the presence of activating protein in both. One can suggest the involvement of structural changes in chromatin. Actually, the test for DNA methylation showed the absence of *mts1* methylation in CSML-100, while in CSML-0 cells the *mts1* gene was heavily methylated. The latter may interfere with binding of activating proteins to the enhancer. The effect should be indirect as the enhancer core sequence does not contain CpG dinucleotides.

The second cis-regulatory element is represented by the sequence TGACTCG differing from the consensus AP-1 binding sequence (TGACTCA) by one base substitution. As a result of substitution, a CpG dinucleotide appears that is the subject for deoxycytidine methylation. Both methylated and non-methylated sequences interact with nuclear proteins, as followed from band shift experiments. The protein binding to methylated sequence is different from that binding to non-methylated one and is much more abundant in nuclear extracts. The consensus AP-1 binding sequence competes only with the methylated element. This suggests that only the methylated sequence binds AP-1 factor. The conclusion was proved in supershift experiments with antibodies to Jun and Fos proteins. Thus, methylation of CpG creates a novel site for AP-1 binding. In the *mts1* gene, methylated AP-1 binding element plays the role of a transcription silencer of a moderate strength. This inhibition seems to play a role *in vivo*, as a particular CpG sequence in CSML-0 cells is not methylated, while in CSML-0 cells, it is completely methylated (Tulchinsky *et al.* 1996).

The third cis-regulatory element is located further downstream and is represented by the GGGGTTTTTCCAC sequence, related to NFκB-binding sites. The sequence does actually bind NFκB (p50/p50 and p50/p65), but this binding does not change *mts1* transcription, at least in experiments with transient expression. On the other hand, the same sequence binds another factor, p200, of a higher molecular weight. As was shown in experiments with different constructs, the latter was responsible for activation of transcription in transient transfection assays. The p200 concentration in CSML-100 extract is about tenfold higher, than that in CSML-0 cells. *In vivo* footprinting showed the occupancy of the DNA sequence only in CSML-100 cells. These data suggest the role of the factor in the *in vivo* activation of *mts1* transcription (Tulchinski et al., 1997). Closely to the previous element, a fourth element is located, which contains a microsatellite motif and is protected from nucleases in both *in vivo* and *in vitro* footprinting assays. It binds a protein interacting with microsatellite. It seems to play *in vivo* a role of a moderate transcription activator, as follows from stable transfection experiment (Prokhourtchuk et al., in preparation).

The fifth positive cis-regulatory element binds a novel protein which is present only in CSML-100 cells. Therefore, it may represent a metastasis-specific transcription factor. Its cloning is in progress. Finally, the sixth regulatory element is represented by the enhancer binding AP-1 protein. In CSML-100 it is a FRA-JunD

Table 3. Results of transfection experiments with the *mts1* gene constructions

Cells	Phenotype	*mts1* construction	Mts1 in transfected cells	Change of metastatic phenotype
CSML-100	**M**	Antisense	**Decrease**	**Strong decrease**
OHS (human)*	**M**	Ribozyme	**Decrease**	**Strong decrease**
CSML-0	**NM**	Sense	Absence	No change
Line 1 + DMSO	**NM**	Sense	**Appearance**	**Strong increase**
Rama 37 (rat)**	**NM**	Sense	**Appearance**	**Strong increase**
MCF-7 (human)	**NM**	Sense	**Appearance**	**Increase**
Transgenic mice; spontaneous adenocarcinoma	**NM**	Sense	**Appearance**	**Strong increase**

complex. CSML-0 cells are FRA-deficient, and this may play an important role in the transcription control.

Thus, the first intron of the *mts1* gene contains a complex regulatory system, which is sensitive to methylation. Some factors present in this system may play an important role in the control of metastatic behaviour of tumors.

B. The role of *mts1* expression in tumor metastasis.

The central question is whether the over-expression of the *mts1* gene in tumor cell can or can not change the phenotype from non-metastatic to metastatic and *vice versa*, i.e. whether the presence of Mts protein is casual for metastatic behavior of the tumor cell or an occasional coincidence. Certainly, the over-expression of any cellular gene could not be expected to constitue the only factor responsible for metastasis (see above), but some genes on certain background of expression of other genes may become indispensable for that. These "key genes" can be detected in transfection experiments that allow to switch on or off the gene functioning.

Several cell systems were used in such experiments (**Table 3**). First, CSML-100 cells were transfected with the construct containing *mts1* cDNA in antisense orientation under the control of Moloney sarcoma virus promoter/enhancer element present in its LTR. The cell lines actively expressing antisense RNA were selected and used for subcutaneous transplantation to isogeneic mice.

They had a dramatically decreased metastatic potential compared to highly metastatic CSML-100 cells. Instead of hundreds of metastatic foci expected in the lungs of mice subcutaneously injected with the original CSML-100 cells, either no foci or single metastases appeared after transplantation of the same cells but expressing *mts1* antisense RNA. Thus, *mts1* expression was necessary for maintaining a metastatic phenotype in CSML-100 cells (Grigorian *et al.* 1993).

Another technology to switch off the gene expression is to use a construct encoding ribozymes, i.e. RNAs that specifically cleave a particular RNA. The ribozyme specifically cleaving human *mts1* RNA at the second exon was transfected into human osteosarcoma (OHS) cells. The control OHS cells gave metastases to bone marrow of nude rats after intracardiac injection. The stable transfectants strongly suppressed the metastatic phenotype. The Mts1 protein content in such cells was decreased (Maelandsmo *et al.* 1996).

The reverse experiment with CSML-0 cells stably transfected with a construct expressing sense *mts1* mRNA gave negative results. However, in spite of active *mts1* transcription, these cells did not contain Mts1 protein. Thus, in addition to the control of *mts1* expression at the transcription level, the control at translational level is also important and some cells can not translate *mts1* mRNA. Therefore, these experiments are non interpretable until the translation suppression is overcome.

Yet sense constructs were successfully tested in three other cell lines. One of them is line 1 cells, that are mouse small cell lung carcinoma cells highly metastatic to lungs upon intravenous injection. However, after dimethylsulfoxide (DMSO) treatment, they lose the ability to metastasize. DMSO treatment was also shown to strongly inhibit *mts1* expression. Sense constructs were transfected to these cells and the transfectants, actively expressing exogenous *mts1* gene, acquired the ability to give metastases even after DMSO treatment. The latter did not interfere with *mts1* expression governed by MSV-LTR control elements (Grigorian *et al.* 1993).

A strong increase in metastatic potential was found in rat mammary epithelial Rama37 cells after stable transfection with the *mts1*-expressing constructions. The original cells are benign and do not metastasize, while transfectants gave metastases to lungs and lymph nodes (Davies *et al.* 1993).

Finally, experiments on the well-characterized human mammary adenocarcinoma MCF-7 cells were performed.

MCF-7 cells are rather benign. They can grow after transplantation to nude mice only when supported with estrogen and only when transplantation into the mammary fat pad is performed. The growth is non-invasive and no metastases can be observed. MCF-7 cells do not contain any significant amount of Mts1 protein. Only a low level of *mts1* expression could be observed in stromal cells. The expression of the exogenous *mts1* gene induced by stable transfection with the construct containing the *mts1* gene strongly changed the properties of the MCF-7 cell growth. First, their growth in nude mice became hormone-independent. Second, they could grow after just subcutaneous transplantation. Third, an invasive growth at the primary focus could be detected. Fourth, metastases to regional lymph nodes and small-size metastases to the lungs were observed. The tumor cells contained varying amounts of Mts1 protein (Grigorian *et al.* 1996). Thus, in all mentioned cases, the appearance or disappearance of Mts1 protein led to a significant modulation of metastatic phenotype in the expected direction.

Yet, a weak point in transfection experiments is the heterogeneity of the cell population used for transfection. For example, CSML-0 cells consist of three morphologically distinct cell types that are reproduced after cloning from individual cells. It can not easily be excluded that only cells with pre-existing differences in metastatic potential have been selected during transfection experiments. Some other approaches should also be used.

The most clear evidence for the casual role of the *mts1* gene expression for creation of metastatic phenotype was obtained in experiments with transgenic animals (Ambartsumian *et al.* 1996) (**Fig. 13**). Transgenic mice were obtained with the construct containing the *mts1* gene under control of the MMTV-LTR promoter/enhancer

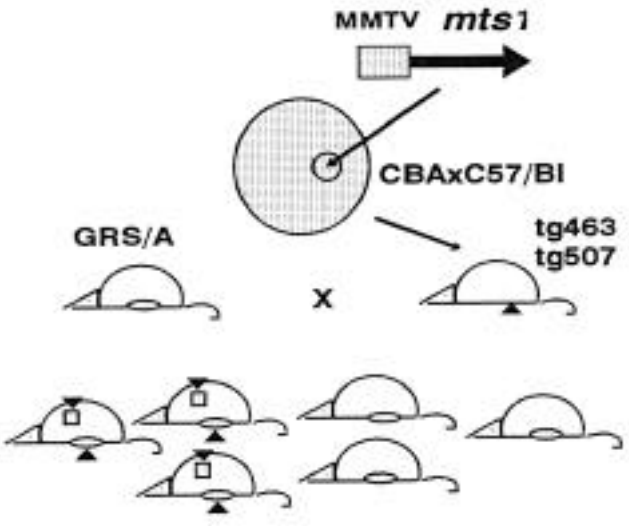

Fig. 13. The scheme of the experiment with transgenic animals.

(❍), mammary adenocarcinomas; (□), metastases; (▲) presence of Mts1 protein.

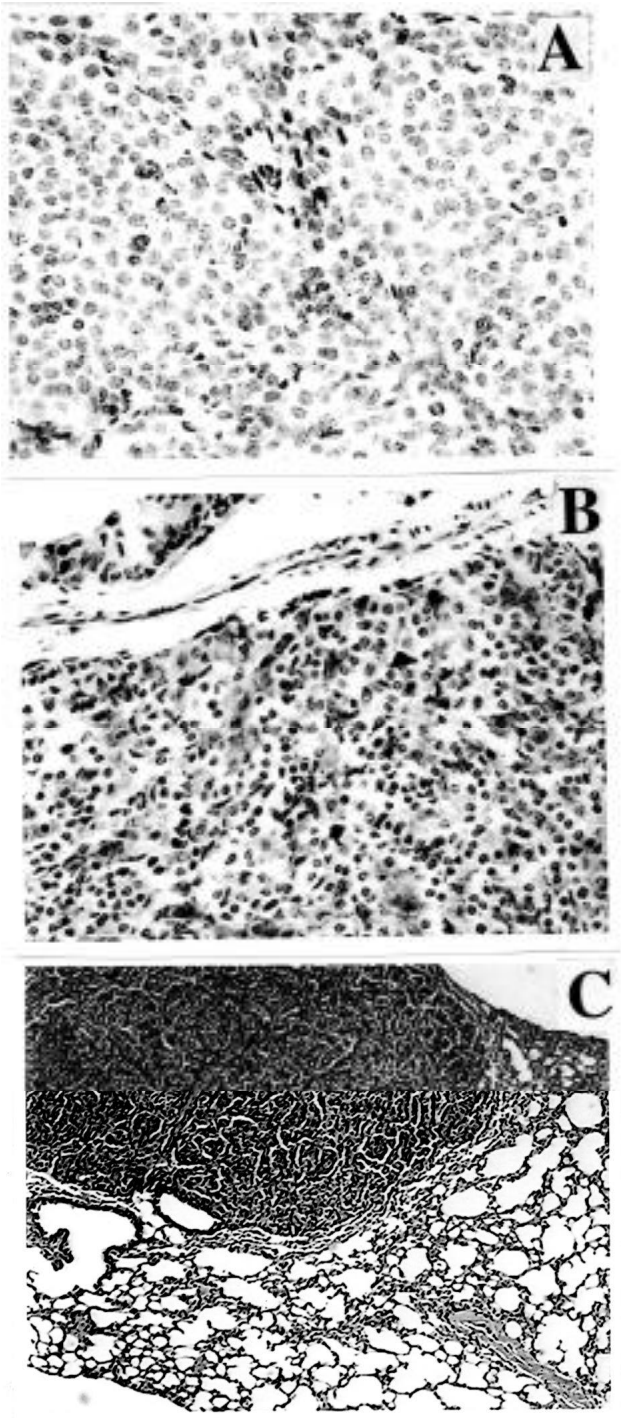

Fig. 14. Staining of the non-transgenic (**A**) and transgenic tumor (**B**) with antibodies to Mts1 and the metastasis of transgenic tumor to the lungs (**C**).

Table 4. Appearance of metastases in spontaneous and transplanted tumors depending on the presence of actively expressed *mts1* transgene

Type of the tumor	Mice line and generation	Number of tumors	Number of tumors with metastases
Spontaneous mammary adenocarcinoma	Tg463 (F1-F4)	23	9 (39%)
	Tg507 (F1)	5	2 (40%)
	Transgenic total	**28**	**11 (40%)**
	Non-transgenic	21	1 (5%)
Transplantation to nude mice	Transgenic metast.	5	3
	Transgenic non-met.	2	2
	Transgenic total	**7**	**5 (70%)**
	Non-transgenic	**2**	**0 (0%)**

element. Transgenic mice expressed exogenous *mts1* in several tissues. The highest level of expression was found in lactating mammary glands, where the MMTV promoter is very active. The endogenous *mts1* gene is not expressed in lactating mammary gland. Interestingly, the phenotype of transgenic mice was not changed compared to normal mice. Even the presence of a high amount of Mts1 protein in lactating mammary glands did not interfere with their functioning and no mammary gland tumors could be observed. Obviously, the *mts1* gene is not an oncogene.

Thereafter, the transgenic mice were crossed with mice from the GRS/A strain characterized by a high incidence of mammary gland tumors appearing after several cycles of pregnancy and lactation. These tumors are non-metastatic. As transgenic mice were heterozygous, only half of the offspring carried the transgene, while another half represented the control group. The tumors appeared with the same high frequency in both groups and they were morphologically indistinguishable. The tumor growth rate also did not depend on the presence of the transgene.

However, a dramatic difference in the metastatic potential of tumors from the two groups was found. As was mentioned above, non-transgenic tumors never metastasize. Just in only one case (out of 21 tested), non-transgenic tumor gave metastases to lungs, but this probably depended on certain additional genetic changes. On the other hand, 40% of transgenic tumors were metastatic (**Table 4**). Then, the tumors were subcutaneously transplanted to athymic mice to determine their metastatic phenotype. Both transgenic tumors that had metastasized before and transgenic non-metastasizing tumors gave rise to lung metastases. Thus, 40-50% incidence of metastasis is an intrinsic feature of spontaneous mammary carcinomas expressing the *mts1* gene. Non-transgenic tumors never metastasized after transplantation, with the above mentioned exception.

The distribution of Mts1 protein was detected with the aid of immuno-staining (**Fig. 14**). Mts1 was found in transgenic tumor cells at the primary focus as well as in the metastatic foci. The concentration varied in a wide range among different cells even in the same tumor. Non-transgenic tumor cells in neither case contained Mts1 protein. The stromal cells in the both types of tumors contained the same small amounts of Mts1 expressed from endogenous gene.

All mentioned experiments clearly demonstrate that in some tumors *mts1* expression is necessary and sufficient for the acquisition of the metastatic phenotype. It is quite clear that *mts1* can not be responsible for all metastatic phenotypes, as several metastatic tumors, in particular one which appeared among the non-transgenic tumors, do not express *mts1*. However, the described results show that the *mts1* gene is one of the key metastatic genes. The question arises, what is a possible mechanism of the action of Mts1 protein.

C. A possible biological role of the Mts1 protein.

To answer the question, intensive studies of the protein had to be performed. For this, one needs high amounts of protein. It was obtained in a bacterial system with oligohistidine tail that allowed an easy purification of the protein on nickel columns. The purified protein was used for preparing polyclonal and monoclonal antibodies.

Western blot analysis and immunostaining of cells with these antibodies showed the Mts1 protein to be localized in the cytoplasmic fraction, like other calcium-binding proteins. Experiments on fractionation of cell extracts suggested the presence of a significant fraction of Mts1 protein in the cytoskeleton.

To understand Mts1 function, attempts to determine the targets of Mts1 protein were performed. The *in vivo* labeled proteins which bind Mts-1 were immunoprecipitated with antibodies to Mts1 and the proteins specifically precipitated by these antibodies were

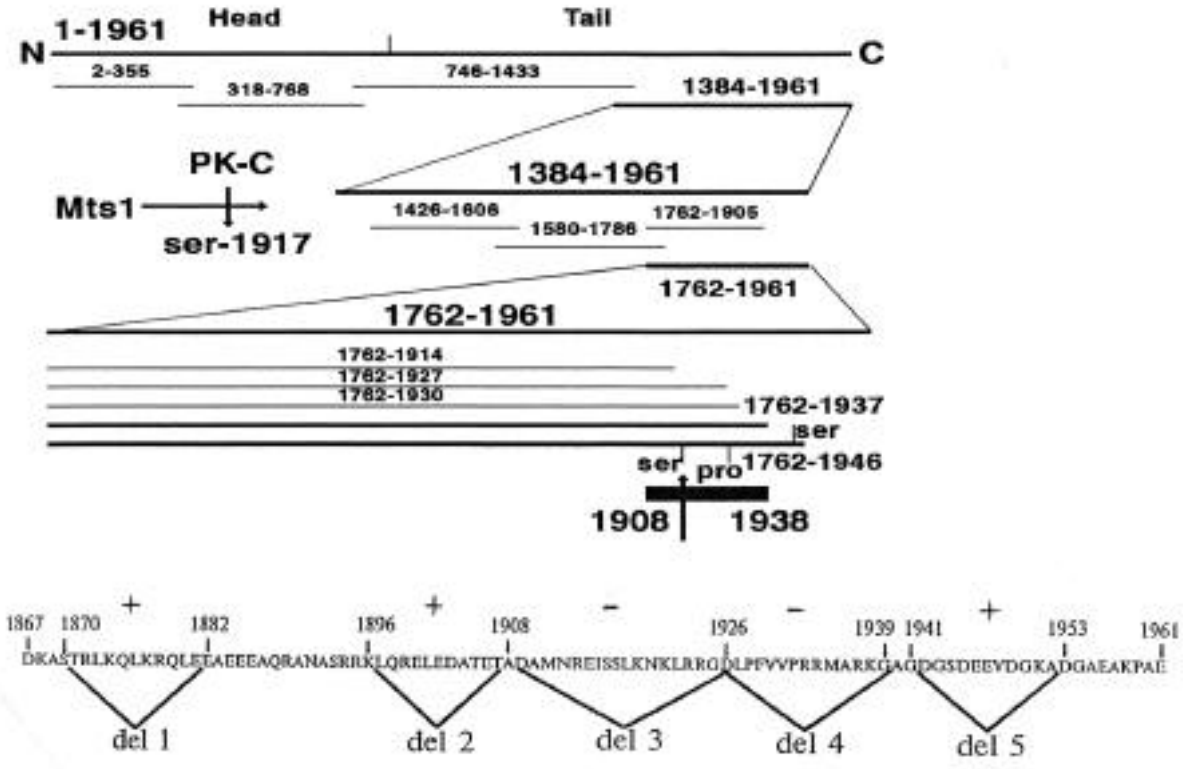

Fig. 15. Interaction of Mts1 with carboxy-terminus of heavy chain of non-muscle myosin.

analyzed. This approach resulted in the isolation of different components of myosin complex. On the other hand, antibodies to myosin co-precipitated Mts1. Ultracentrifugation of cell extracts on sucrose gradients also demonstrated cosedimentation of a rather significant fraction of Mts1 with a much heavier myosin complex suggesting their reversible association. After double immuno-staining with antibodies to Mts1 and myosin, one can see the exact coincidence in fluorescence distribution for both antibodies. Mts1 interacts only with the heavy chain of non-muscle myosin as followed from different overlay experiments and using antibodies specific for different types and different chains of myosin. Mts1 does not interact with the light chain and with heavy chain of smooth muscle myosin (Kriajevska *et al.* 1994).

To further analyze Mts1-myosin interaction, different fragments of the heavy chain of non-muscle myosin (HCNMM) were prepared using recombinant DNA and tested for interaction with Mts1 in protein overlay experiments. Only the carboxy-terminal part of HCNMM did react. Analysis of the deletions obtained in this part of HCNMM showed that the only peptide responsible for interaction with Mts1 was located between the 1908 and 1938 aminoacid residues (**Fig. 15**). Then the effect of such binding on protein kinase mediated phoshorylation of heavy chain of non-muscle myosin was tested. Mts1 specifically inhibited phoshorylation of serine residue no. 1917 by protein kinase C without any effect on other phosphorylation sites and without interference with casein protein kinase action. The target serine residue is located just inside the binding region for Mts1 (**Fig. 15**). One can suggest that at least one of Mts1-induced effects is an inhibition of this phosphorylation reaction. The latter was claimed to play a role in non-muscle myosin functioning putatively leading to changes in cell motility. This may be a possible way for changing the metastatic phenotype of tumor cells. However, for the time being, this is just a hypothesis.

Another consequence of interaction between Mts1 and carboxy-terminal part of non-muscle myosin is the solubilization of the non-muscle myosin. The simple addition of a native Mts1 to the myosin polypeptide precipitate at physiological salt concentration completely solubilized the precipitate, confirming the role of Mts1 in myosin disaggregation.

It should be pointed out that myosin is not the only target for Mts1. In particular, the method of binding to affinity column revealed another protein, p37, interacting with the Mts1 column. The p37 protein binds to Mts1 in a calcium-dependent manner. Interestingly, this binding

strongly changes the interaction of Mts1 with calcium. Two calcium-binding domains in Mts1 act cooperatively and in general the affinity to calcium is increased. Possibly, p37 may play some role in the control of Mts1 functions connected with binding of calcium ions (Dukhanina *et al.* in press).

Another important function of Mts1 protein may be excerted via mesenchymal transformation of epithelial cells. Strutz *et al* (1995) found, that expression of the *mts1* gene in epithelial cells might induce their mesenchymal transformation. We have found that appearance of Mts1 proteins in the cells of transgenic mice was accompanied by the loss of E-cadherin. The reverse correlation between Mts1 and E-cadherin content may be especially important as E-cadherin is one of most clear-cut "antimetastatic proteins" with well understood function (Vlamincks *et al.* 1991). The mechanism of Mts1 influence on E-cadherin remains unclear.

Finally, Onishchenko et al. (1997) recently observed the destabilization of blood vessels in tumors expressing *mts1*; this means that endothelial cells of blood vessels may constitute another target of Mts1 and this additional phenotypic effect of Mts1 may be effected through its influence on tumor vascularization and on the state of endothelial cells.

Thus, Mts1 may be a multifunctional regulator of cell functions connected with the acquisition of an invasive metastatic phenotype. The *mts1* gene may be one of the critical genes for metastasis development and, therefore, a promising target for cancer gene therapy.

VIII. Conclusions

In general, this study summarizes our results on the search for new genes and proteins controlling tumor progression. Here we described explicitly two examples of such genes. One gene, (*mts1*), tentatively assigned to the second class (tumor progression genes), and another gene, (*tag7),* putatively belonging to the third class (genes for biological defense against tumors) were discovered in this research program together with many other, yet less characterized genes. The *tag7* gene seems to be a promising factor to be used for gene therapy or even for the direct treatment of tumors by its product, Tag7 protein. We propose that the *mts1* gene may also find a place in the constellation of target genes for cancer gene therapy.

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