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**Contribution of the Receptor/Ligand Interaction between CD44 and Osteopontin to Formation of Breast Cancer Metastases**

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**Cancer is characterized by dysregulated growth control, overcoming of cellular aging, and metastasis formation. The latter distinguishes malignant tumors from benign tumors and is mediated by groups of molecules called homing receptors, cytokines, and proteinases. Expression of these molecules on tumor cells determines when and where particular types of cancer spread.**

**The physiologic role of the relevant receptors, cytokines, and proteases in the healthy, cancer-free organism has been incompletely understood.** We have studied a homing receptor, known as CD44, and its ligand, the cytokine osteopontin. In host defense, CD44 and osteopontin play key roles in mediating delayed types of immune response that are important in tuberculosis, organ transplantation, and many forms of vaccination. Macrophages are the cells that mainly determine whether an immune reaction will have delayed (cell-mediated) or acute (antibody-mediated) characteristics, and osteopontin and CD44 direct macrophages to the former. The engagement of CD44 by osteopontin also induces macrophage migration, a mechanism that metastatic tumors may utilize in the process of dissemination. We have found that other gene products that contribute to dissemination of cancerous cells similarly contribute to host defenses, and we conclude that metastasis genes are developmentally nonessential genes which physiologically mediate stress responses, inflammation, wound healing, and blood vessel formation.
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INTRODUCTION

In November 1999, I left a basic immunology department at the Dana-Farber Cancer Institute and joined the Division of Radiation and Cancer Biology at the New England Medical Center and Tufts University Medical School which is headed by the renowned breast cancer researcher Dr. Vimla Band. This environment will provide me with more fruitful interactions revolving around breast cancer and with ample resources for breast cancer research. As an inevitable consequence of the transition, however, this report also reflects the need to bring old projects to completion (some of the previously ongoing studies are published or in press) and a delay in setting up a new laboratory (progress in year 2 was less extensive than in the previous budget period).

Splice variants of the homing receptor CD44 mediate metastasis formation by various tumors. Expression of the cytokine osteopontin has been associated with malignant potential of tumor cells. We have identified a novel receptor/ligand interaction between CD44 and osteopontin that mediates cell attachment or migration. Both molecules are subject to multiple posttranscriptional and posttranslational modifications and our data suggest that these alterations modulate binding. Organ-preference in metastasis formation may be based on specific recognition via homing receptors and modifications in CD44/osteopontin interaction may provide a potential molecular explanation. To assess why breast cancer metastasizes predominantly into bone, we are investigating modifications in both proteins that allow interaction.
Specific Aim 1: Identification of the posttranslational modifications in both proteins which are permissive for interaction.

Tasks 1-3: We had previously performed structure activity analyses of osteopontin and its receptors in macrophage migration and activation as a model for metastatic spread and found that an interaction between the C-terminal domain of osteopontin and the receptor CD44 induces macrophage chemotaxis via G-protein signaling, while engagement of integrin receptors by a non-overlapping N-terminal osteopontin domain induces cell spreading, mediated by PKC, and macrophage activation involving phosphatidylinositol as a secondary signal transduction component. Serine phosphorylation of the osteopontin molecule on specific sites is required for functional interaction with integrin but not CD44 receptors. A revised version of the manuscript detailing the structure activity analysis of osteopontin has recently been submitted to the European Journal of Immunology, while the associated signal transduction pathways are still subject to further study.

Task 4: Gene targeted osteopontin−/− mice fail to develop delayed type (cellular) immune responses or Th1-driven autoimmunity after viral infection. This defective immune activation is associated with diminished production of the stimulatory cytokine IL-12 and excessive production of the inhibitory cytokine IL-10. A phosphorylation-dependent interaction between the N-terminal portion of osteopontin and its integrin receptor on macrophages leads to IL-12 expression, while a phosphorylation independent interaction of osteopontin with CD44 inhibits IL-10 expression. This manuscript has now been published in the journal Science (Ashkar et al. 2000; see appendix). Based on the immunomodulatory effect of osteopontin, we have designed candidate peptide drugs that selectively interfere with receptor binding to either CD44 or integrin αvβ3 (patent pending; see appendix).

Cancers of particular tissue origin show consistent preference for specific target organs to spread to.
This topology of metastasis formation is mediated by the potpourri of homing receptors on the
tumor cell surface and their ligands and is widely believed to have its physiologic correlate in
morphogenesis during embryonic development. Unexpectedly, knockout mice in which individual
genes known to participate in tumor spread were disrupted turned out to be fertile and
developmentally normal. The defects observed in the relevant gene targeted mice are impairments
of various features of stress responses. Metastasis-associated gene products have several
characteristics in common. They comprise a set of developmentally non-essential genes which
physiologically mediate stress responses, including inflammation, wound healing, and
neovascularization. Recognition of topology is encoded in the surface molecules of immune cells
and organ preference by cancer may be derived from a process which immune cells use to target
their responses. Metastasis-associated gene products therefore constitute a unique and essential
group of cancer related biomolecules. We conclude that metastasis formation is a process that
mimics macrophage behavior (Weber/Ashkar 2000a,b; see appendix). We believe that this insight
constitutes a major paradigm shift for two reasons 1) the classical cancer related genes (oncogenes,
tumor suppressor genes, telomerase, and DNA repair genes) do not account for metastasis
formation, 2) currently, most metastasis researchers favor the assumption that metastasis formation
copies mechanisms of organ development; this notion, however, is clearly refuted by the absence of
developmental abnormalities in the relevant knockout mice.
KEY RESEARCH ACCOMPLISHMENTS

* structure-activity analysis of osteopontin in cell migration and activation

* identification of suppression of IL-10 expression by macrophages as consequence of interaction between osteopontin and CD44 which may have implications for tumor immunology

* theoretical consideration regarding metastasis formation as mimicry of macrophage activation
REPORTABLE OUTCOMES


Weber GF, Ashkar S. Stress response genes - the genes that make cancer metastasize. Journal of Molecular Medicine 2000, in press. attached

CONCLUSIONS

We have further delineated the functional consequences of the interactions between osteopontin and its receptors. We have identified the mechanism of cell motility as a two-step process involving both major osteopontin receptors. These may be the molecular underpinnings of tumor invasion. We have further characterized the important involvement of osteopontin in delayed type immune responses which may have implications for tumor immunology. Clarification of the physiologic roles of osteopontin and its receptors has enabled us to propose fundamental features that are shared by all metastasis mediating gene products.
REFERENCES


Eta-1 (Osteopontin): An Early Component of Type-1 (Cell-Mediated) Immunity

Eta-1 (Osteopontin): An Early Component of Type-1 (Cell-Mediated) Immunity

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Cell-mediated (type-1) immunity is necessary for immune protection against most intracellular pathogens and, when excessive, can mediate organ-specific autoimmune destruction. Mice deficient in Eta-1 (also called osteopontin) gene expression have severely impaired type-1 immunity to viral infection [herpes simplex virus–type 1 (KOS strain)] and bacterial infection (Listeria monocytogenes) and do not develop sarcoïd-type granulomas. Interleukin-12 (IL-12) and interferon–γ production is diminished, and IL-10 production is increased. A phosphorylation-dependent interaction between the amino-terminal portion of Eta-1 and its integrin receptor stimulated IL-12 expression, whereas a phosphorylation-independent interaction with CD44 inhibited IL-10 expression. These findings identify Eta-1 as a key cytokine that sets the stage for efficient type-1 immune responses through differential regulation of macrophage IL-12 and IL-10 cytokine expression.

The development of cell-mediated (type-1) immune responses is necessary for protection against the growth of many infectious pathogens and, when excessive, can mediate autoimmune host tissue destruction. Although macrophage activation by microbial pathogens (1, 2) and foreign body reactions (3) are associated with type-1 immunity, the cellular and molecular events that imprint this response are not fully understood. An essential early step in this process is macrophage production of IL-12 at sites of infection, whereas early IL-10 production inhibits this response (4). Although IL-12 responses can be triggered by an interaction between the CD40 ligand on activated T cells and CD40 on macrophages (4), this interaction also induces the inhibitory IL-10 cytokine (5, 6), and its transient nature may not suffice for sustained IL-12 induction in vitro (7) or in vivo (8).

A gene product that may play an important role in the development of type-1 immunity is the T cell cytokine Eta-1 (for early T lymphocyte activation–1), also known as osteopontin (Opm) (9). The Eta-1 gene is expressed in T cells early in the course of bacterial infections (within 48 hours), and interaction of its protein product with macrophages can induce inflammatory responses (10). Genetic resistance to infection by certain strains of Rickettsia may depend on Eta-1–dependent attraction of monocytes into infected sites and acquisition of bacterioidal activity (11); the granulomatous response characteristic of sarcoidosis and tuberculosis are associated with high levels of Eta-1 expression (12).

Granuloma formation in these human diseases is a cellular consequence of type-1 immunity (12), and sarcoïd-type granulomas can be induced in mice after injection of polyvinyl pyrrolidone (PVP) (13). Because certain murine models of parasite-induced granulomas may reflect a mixture of type-2 and type-1 immunity (6), we first established the importance of IL-12–dependent type-1 immunity in this murine model of granuloma formation. An intense granulomatous response was provoked shortly after (subcutaneous) injection of PVP into C57BL/6 (+/+ ) but not C57BL/6 naïve strains of mice. This response was diminished by 70 to 80% in C57BL/6 IL-12–/– mice and was enhanced two- to threefold in C57BL/6 IL-10–/– mice (Fig. 1, A and B). Because C57BL/6 naïve mice injected with PVP and purified Eta-1 displayed a granulomatous reaction, this gene product can partially substitute for activated T lymphocytes in this setting (Fig. 1, A and B).

We then asked whether mice deficient in Eta-1 secondary to targeted gene mutation (14) formed granulomas after PVP injection. Eta-1–/– mice did not develop a detectable granulomatous response after challenge with PVP; the response was partially restored by coinjection of purified Eta-1 with PVP (Fig. 1, A and B). Histologic analysis of granulomas formed in Eta-1–/– mice and in Eta-1–/– mice reconstituted with purified Eta-1 revealed a similar macrophage–dominant cellular infiltrate: About 85% of granulomatous cells in both cases were Mac-1+, whereas 5 to 10% were CD3+ T cells or B220+ B cells. BP-35+ neutrophiles, which were only a minor component (1 to 2%) of granulomas in these mice, increased 5- to 10-fold in the granulomas of IL-10–/– mice (Fig. 1C). Eta-1–/– mice also displayed defective granulomatous responses to injection of collagen and latex, consistent with reports that human T cells resident in sterile granulomas have high expression of Eta-1 (12). Restimulation of lymph nodes draining subcutaneous sites of PVP injection in Eta-1–/– mice and control mice with PVP revealed impaired IL-12 and interferon–γ (IFN-γ) responses: The IL-12 response was reduced by nearly 95%, and the IFN-γ response of Eta-1–/– mice was reduced by 90% in comparison to Eta-1+/+ controls (Fig. 1D).

We next defined the role of Eta-1 in the immune response to herpes simplex virus–type 1 (HSV-1) (KOS strain) infection. Eta-1–/– mice infected by HSV-1-1×106 plaque-forming units (PFU) via the cornea did not develop a significant tuberculin-type delayed-type hypersensitivity (DTH) response after footpad challenge with HSV-1-106 PFU, in contrast to the strong DTH response of Eta-1+/+ controls (Fig. 2A, left). Although the numbers of T cells and proportions of T cell subsets in the thymus and peripheral lymphoid tissues of Eta-1–/– mice were similar to Eta-1+/+ littermates (15), defective antiviral DTH responses might reflect a subtle alteration in lymphocyte or macrophage development. We therefore tested the effects of acute in vivo depletion of Eta-1 with a neutralizing antibody. Administration of antibody to Eta-1 (LF-123) (16) immediately before and repeatedly after HSV-1 infection efficiently inhibited the DTH response upon rechallenge (Fig. 2A, right).
lation in vitro (19). However, draining lymph node cells from virally infected Eta-1−/− mice produced exaggerated amounts of IL-10 and IL-4 and reduced IL-12 in comparison with Eta-1+/+ controls (Fig. 2C). In contrast with this sterile granulomatous response, IFN-γ levels were not reduced in Eta-1−/− mice after HSV-1 viral infection (20), consistent with an IL-12–independent pathway to IFN-γ production that may depend on virally induced IFN-α/β production (21).

We then investigated the ability of Eta-1−/− mice to mount a protective immune response after bacterial infection. The murine response to Listeria monocytogenes is an experimental cornerstone of our understanding of the early events leading to type-1 immunity after microbial infection (1) and depends on early macrophage production of IL-12 and downstream expression of IFN-γ (22). Eta-1−/− mice were defective in their ability to clear L. monocytogenes after systemic infection, similar to the defect in IL-12−/− mice (23) (see Web figure 1, available at www.sciencemag.org/feature/data/1046451.shl). Restimulation of spleen cells from Eta-1−/− and Eta-1+/+ mice with heat-killed bacteria revealed that cells from the former mice had reduced IFN-γ responses: 25.5 ± 6.5 ng/ml of IFN-γ were produced by spleen cells from Eta-1+/+ mice in comparison with 3.2 ± 1.2 ng/ml of IFN-γ from Eta-1−/− mice (24).

Thus, Eta-1 expression may affect type-1 immunity through regulation of the IL-12 and IL-10 cytokine ratio. To define the effect of Eta-1 on IL-10 and IL-12 production by macrophages in vitro, we incubated resident peritoneal macrophages with increasing concentrations of purified Eta-1 in serum-free medium (Fig. 3A). This resulted in the secretion of as much as 400 pg/ml of IL-12 at 48 hours, whereas IL-10 production was not detected (Fig. 3A and B). The failure of Eta-1 to induce IL-10 was somewhat surprising because other cytokines that activate macrophages, including tumor necrosis factor–α, IL-1, IL-2, IL-3, and IL-6, all stimulate IL-10 secretion (25), and lipopolysaccharide (LPS) stimulation of these resident peritoneal macrophages induced both IL-12 (~250 pg/ml) and IL-10 (~100 pg/ml) (Fig. 3B). Further analysis showed that Eta-1 actively suppressed the LPS-dependent IL-10 response of resident peritoneal macrophages by 80 to 95% (Fig. 3C).

The interaction of Eta-1 with macrophages is mediated through two functional receptors. Engagement of CD44 mediates chemotactic migration (26), and interaction with α2β1 integrin causes haptotaxis, adhesion, and spreading (10, 27). We asked which receptor was responsible for the regulation of macrophage cytokine production by Eta-1. Induction of IL-12 is inhibited by GRGDS (28) peptide (but not GRADS peptide) (29) and by antibody to the integrin β1 subunit (but not by antibody to CD44), and macrophages from CD44−/− mice

![Image](image-url)

**Fig. 1.** Granuloma formation in cytokine-deficient mice. Granulomas were measured 5 days after subcutaneous injection of PVP into mice carrying the indicated mutations of cytokine genes (30). (A) Results are expressed as the mean number of granulomas per high-power field (HPF) (×200 magnification) (left); as the mean number of cells per granuloma after examination of 50 to 80 HPF per mouse (middle); and as the product of these two indices, shown as granuloma burden (right). Error bars indicate 1 SEM. (B) Photomicrographs at indicated magnifications showing histologic analysis of tissue sections at PVP injection sites of Eta-1−/− (left) or Eta-1+/+ (right) mice showing granulomatous infiltrations of mononuclear cells in subcutaneous dermal and subdermal areas 5 days after injection of PVP, PBS, or PVP + 5 μg purified Eta-1 (40). (C) Analysis of surface antigens expressed by cells within granulomas in the indicated mouse strains was done with monoclonal antibodies to Mac-1, B220, CD3, and BP-35 (a neutrophil marker). Error bars indicate 1 SEM. (D) Cytokine expression by cells from lymph nodes draining the site of granulomas 5 days after PVP injection was measured 48 hours after incubation with PVP (2 × 106 cells per well). Data are representative of three independent experiments.
display an unimpaired IL-12 response (Fig. 4, A to C). Moreover, Eta-1−/− dependent induction of IL-12 secretion from macrophages was not due to contamination with endotoxin. *Lactococcus* lysate analysis indicated that purified Eta-1 contained <1 ng/g of endotoxin, and the IL-12 response of macrophages from C3H/HeJ mice (which are defective in endotoxin receptor-mediated signaling) was not obviously impaired in comparison to other strains (Fig. 4C). In contrast to IL-12 induction, inhibition of IL-10 depends on engagement of the CD44 receptor. Eta-1−/− dependent inhibition of IL-10 is blocked by antibody to CD44 but not by antibody to integrin β2, and macrophages from CD44−/− mice are resistant to Eta-1 inhibition of the IL-10 response (Fig. 4, A to C). To further characterize the RGD-dependent interaction with the macrophage integrin receptor, we analyzed fragments from an Eta-1 Lys-C digest and identified a proteolytic fragment from the NH2-terminal portion of Eta-1, which contains the integrin binding site (termed NK10) that is sufficient to induce macrophage IL-12 expression (Fig. 4A).

Eta-1 is secreted in nonphosphorylated and phosphorylated forms (31). Phosphorylation may allow Eta-1 to associate with the cell surface rather than the extracellular matrix (32), through a contribution to integrin binding. In contrast, serine phosphorylation of recombinant Eta-1 is not required for CD44-dependent interactions leading to chemotactic migration (26). We investigated whether phosphorylation of Eta-1 might affect its ability to regulate IL-12 and IL-10 expression. Dephosphorylation of purified, naturally produced Eta-1 abolished IL-12 stimulatory activity; phosphorylation of recombinant Eta-1 at specific sites restored activity (33) (see Web figure 2, available at www.sciencemag.org/content/1046451.sih). Although recombinant Eta-1−/− lacking phosphate groups could not induce IL-12, this molecule retained inhibitory activity for the macrophage IL-10 response (33). Thus, serine phosphorylation can provide molecular information that regulates the biological activity of a secreted protein.

Our data indicate that expression of Eta-1 represents an essential early step in the pathway that leads to type-I immunity. Previous studies have established the importance of macrophage production of IL-12 in this pathway (1, 4, 22). Our experiments suggest that production of Eta-1 by activated T cells is an essential proximal event that potentiates the macrophage IL-12 response through integrin engagement and dampens the IL-10 response through CD44 engagement, leading to up-regulation of type-I cytokines. The latter inhibitory effect on IL-10 may account for enhanced granulomatous responses of CD44−/− mice (30) and the finding that impairment of the granuloma response noted here is somewhat greater than might be anticipated from the response of IL-12−/− deficient mice (Fig. 1). These findings fill a logical gap in our understanding of the early molecular events that lead to type-I immunity. Although down-regulation of CD40 ligand expression by IFN-γ and soluble CD40 occurs within 24 hours after viral infection, IL-12 is detected in serum over the next 7 to 10 days (8). Our experiments suggest that replacement of the CD40L signal by Eta-1 may potentiate the IL-12 response while dampening the IL-10 activity to allow full maturation of type-I immunity, as judged by cellular responses and expression of downstream effector cytokines such as IFN-γ. The ability of an antigen to induce Eta-1 production after T cell receptor ligation may thus determine the ensuing duration and intensity of type-I immune responses. Eta-1 imprinting of the IL-12 and IL-10 response after appropriate peptide stimulation (34) may also increase the likelihood of autoimmune sequelae as shown here (Fig. 2), through pathways that do not invariably require IFN-γ (35).

Eta-1−/− dependent regulation of two early cytokine checkpoints that dictate development of type-I or type-2 immunity also suggests new therapeutic approaches to several diseases. Eta-1 analogs that mediate CD44-dependent inhibition of IL-10 may inhibit sepsis in burn patients (36), and Eta-1 antagonists may ameliorate the clinical course of bacterial arthritis (37). Engineered forms of...
Fig. 3. Differential regulation of macrophage IL-12 and IL-10 responses by purified Eta-1. (A) Dose-dependent induction of IL-12 (open circles), but not IL-10 (solid circles) production, from macrophages by Eta-1. Resident peritoneal macrophages obtained from C57BL/6 mice (47) were incubated for 48 hours (5 x 10^5 macrophages per milliliter) with purified Eta-1 (40), and IL-10 and IL-12 p70 concentrations in the supernatant were determined by ELISA. Assays were done in quadruplicates, and each point represents the mean and standard error (error bars) of three independent experiments. (B) Time course of IL-12 (open circles) p70 and IL-10 (solid circles) expression by resident peritoneal macrophages (5 x 10^5 macrophages per milliliter) after incubation with 5 nM Eta-1 or LPS (30 ng/ml). Assays were performed in quadruplicates, and each data point represents the mean and standard error (error bars) of two independent experiments. (C) Inhibitory effect of Eta-1 on macrophage IL-10 production. Macrophages were activated with LPS (30 ng/ml) for 1 hour before addition of Eta-1 (5 nM) for an additional 48 hours and consecutive measurement of IL-12 and IL-10 by ELISA. Assays were performed in quadruplicates, and each point represents the mean and standard error (error bars) of two independent experiments.

Fig. 4. Regulation of macrophage IL-12 and IL-10 expression by distinct Eta-1 receptors. (A) Secretion of IL-12 by macrophages is mediated by a 10-kD peptide (NK10) derived from the NH2-terminal fragment of Eta-1 (42) and is inhibited by a blocking antibody to integrin β1 (1 μg/ml), but is unaffected by antibody to CD44 (1 μg/ml). Error bars indicate SEM. (B) Induction of IL-10 production by IL-4 (500 U/ml) in the presence or absence of purified Eta-1 (5 nM) and the effects of antibodies to CD44 [Km81 purified from TIB241 (26)] and the β1 integrin (2C9,2G2, PharMingen) are shown. Error bars indicate SEM. (C) Production of IL-12 and IL-10 in response to Eta-1 by peritoneal macrophages from C57BL/6 mice, C57BL/6 mice that are deficient in CD44 gene expression (C57BL/6-CD44-/-), and cells from C3H. HeJ mice. Mean values and standard errors (error bars) from at least four data points are shown.

Eta-1 that imprint type-1 responses after immunization may also be valuable components of viral and cancer vaccines.

References and Notes
9. R. Patacar, R. A. Saavedra, H. Cantor, Crit. Rev. Immunol. 13, 225 (1993); T. J. de Ritter et al., J. Bone Miner. Res. 13, 1101 (1998); were compared to either Eta-1+/+ littersmates or age-matched C57BL/6 x 129SV mice as controls.
10. Eta-1+/+ litters mates were as follows: C57BL/6 x 129 Eta-1+/+ spleen, 93.7 x 10^6 total cells (30.8% CD3, 15.8% CD4, 11% CD8, and 49.7% B220); C57BL/6 x 129 Eta-1+/- spleen, 82.6 x 10^6 cells (27.8% CD3, 16.8% CD4, 9.0% CD8, and 55.5% B220); C57BL/6 x 129 Eta-1-/- lymph node, 32.0 x 10^6 cells (52.4% CD4, 42.8% CD8, 34.2% CD11b, and 12.0% B220); and C57BL/6 x 129 Eta-1-/- lymph node, 21.9 x 10^6 cells (82.8% CD8, 39.3% CD4, 28.4% CD8, and 11.2% B220). T cells from Eta-1+/+ or Eta-1-/- mice expressed levels of CD44 and CD62 that were not distinguishable.
11. T cells expanded by apoptotic supernatant (50 μg of staphylococcal enterotoxin b) intraperitoneal injection into Eta-1+/- or Eta-1-/- mice was indistinguishable at 3 days. +/+ and /- CD4+ cells (percentage of total spleen) increased from 36.3 to 58.7% in +/+ CD4+ cells increased from 3.2 to 5.5% in /- CD4+ cells increased from 2.3 to 2.6%. /- and +/+ CD4+ cells increased from 2.5 to 2.6%. Expression of IL-2 by lymph node and spleen T lymphocytes from Eta-1+/+ and Eta-1-/- littersmates in response to immobilized antibody to CD3 was also indistinguishable between the C57BL/6 x 129SV Eta-1+/+ and C57BL/6 x 129SV Eta-1-/- mice.
15. HSV-1 stimulation of lymph node cells and splenic macrophages: The right superficial cervical draining lymph node of Eta-1+/- mice and Eta-1+/- littermate controls were harvested 15 days after infection of the right eye with 4 x 10^5 PFU of HSV-1 (KOS). Cells from these lymph nodes (2 x 10^6 cells per well) were incubated in the presence of 4 x 10^7 PFU of ultraviolet (UV)-inactivated HSV-1 (KOS). Supernatants harvested 48 hours later were tested for IL-10 and IL-12 p40 cytokine by sandwich enzyme-linked immunosorbent assay (ELISA) using OptiKill antibody sets (PharMingen, La Jolla, CA). IL-4 was measured after stimulation of draining lymph node cells by plate-bound antibody to CD3.
16. S. Askar et al., data not shown.
19. Listeria infection and cytokine production were followed as described Virulent L. monocytogenes [strain 1778, American Type Culture Collection (ATCC) designation 43252] was grown in Bacto tryptose soy broth (Recton-Dickinson), and 10^6 colony-forming units (CFU),
a sublethal dose for this strain of *L. monocytogenes*, were injected intravenously. The titer of viable bacteria in the inoculum and in organ homogenates was determined by plating 10-fold serial dilutions on tryptic soy agar plates. Eta-1-/- mice contained liver- and splenic *L. monocytogenes* that were apparent 4 to 5 days after infection. Plates were incubated at 37°C, and the numbers of CFU were counted after 24 hours.

24. Spleen cells (4 x 10⁶/ml) from four to five C57BL/6 x 129 Eta-1-/- or four to five C57BL/6 x 129 Eta-1-/- mice that had been intravenously inoculated 5 days earlier with 10⁶ CFU were stimulated with heat-killed *L. monocytogenes* (2 x 10⁶ CFU/ml) 96 hours before RNA measurement by an Optima ELISA kit (Pharmingen).


28. Addition of 5 mM GRGS peptide but not GRADS peptide (29) to cultures of peritoneal macrophages (30) resulted in an 80% reduction of the IL-12 response after LPS stimulation.

29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; and T, Thr.


33. Dephosphorylation of Eta-1 is as follows. Five milligrams of purified Eta-1 was dephosphorylated using 6 U (60 U/mg) of type-II potato acid phosphatase (Sigma) as described (39). Phosphorylation of recombinant Eta-1-glutathione S-transferase (GST)-Eta-1 (5 mg) was phosphorylated as described (39). S. Ashkar, J. L. Schaffer, E. Sallh, L. C. Gerstenfeld, M. J. Glincner, Ann. N.Y. Acad. Sci. 760, 296 (1995); (27). Phosphoamino acid analysis of the recovered protein revealed a phosphoserine content of 16 mol of phosphate per 1 mol of protein and 0.8 mol of phosphothreonine per 1 mol of protein.


38. PVP-dependent granulomas were formed as follows. C57BL/6 (+/+) mice and C57BL/6 nu/nu mice were injected subcutaneously in the right hind limb with 500 µl of 0.3% PVP, C57BL/6 nu/nu and Eta-1-/- mice were injected with this inoculum of PVP and 10 µg of Eta-1 purified as described below. After 5 days, mice were killed, tissue was extracted for histologic analysis, and local lymph nodes were obtained for cytokine expression. Samples were fixed in 10% buffered formalin and embedded in paraffin. 4- to 5-µm serial sections were stained with hematoxylin and eosin, and images were captured with a Sony DVC-970MD video camera and Optima 52 Histomorphometric analysis software.


40. Recombinant GST-Eta-1 fusion protein derived from Escherichia coli was digested with factor Xa and purified by affinity chromatography (S. Ashkar, M. J. Glincner, E. A. Savvedo, Biochem. Biophys. Res. Commun. 214, 274 (1993); (39). Briefly, native Eta-1 was isolated from MC3T3-E1 cells or Ar5V T cells after concentration in PBS using a Millipore tangential flow system, applied to a Millipore LC100 equipped with a DEAEM-Mono HR 1000 cartridge, and developed in a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer (pH 7.4). Eta-1-containing fractions were pooled (the major Eta-1 peak eluted at 0.26 M salt), concentrated by ultrafiltration, chromatographed on Mono P columns (Pharmacia) at pH 8.2, developed with polybuffer 74 (Pharmacia), and eluted from monoabs at pH 4.6. The eluted protein was judged to be pure by several criteria, including SDS electrophoresis and amino acid sequence analysis (NH2-terminal and internal peptide analysis). Mass spectrophotometric analysis revealed a peak centered around a mass of 35,400 daltons that was highly phosphorylated (11 mol of phosphate per 1 mol of protein). O-glycosylated but not N-glycosylated, and without measurable sulfate.

41. Resident peritoneal macrophages obtained by peritoneal lavage with phosphate-buffered saline (PBS) were treated with red cell lysis buffer and incubated (10⁵ macrophages per 100 µl) for 2 hours. The adherent fraction was incubated with 5 nM Eta-1, LPS (30 ng/ml), or recombinant IL-4 (500 U/ml), or as indicated. Supernatant IL-10 or IL-12 p70 was assayed with commercial ELISA kits (R&D Systems, Minneapolis, MN), tested for viability by propidium iodide (>98%), and stained with fluorescein-conjugated antibody to Mac-1 (>98%). Blocking antibody to integrin β2 (from Pharmingen [J. F. Schultz and D. R. Armant, J. Biol. Chem. 270, 11522 (1995)], and antibody to CD44, KM18 (ATCC), was used to block the interaction between CD44 and Eta-1 (16).

42. Although partial tryptic, chemotryptic, or Asp-N endopeptidase digestion of Eta-1 did not reveal an active peptide, a 10-KD fragment isolated from a Lys--C digest (NH2-terminal sequence QETLPSN) was active and predicted to terminate at the thrombin cleavage site. This 10-KD fragment contains ~5 mol of phosphate per 1 mol of peptide at seven potential phosphorylation sites.

43. Supported in part by NIH research grants AI12184, AI37833 (to H.C.), CA76176 (to G.F.W.), and AR43078 (to M.J.G.); grants AR44434, DC10295, ES08897 (to D.T.D.), and CA72740 (to S.R.R.) for development of Opn-null mice at Rutgers University; U.S. Department of Defense breast cancer grants DAMD17-98-1-8060 (to G.F.W.) and DAMD 17-99-1-9124 (to S.A.); U.S. Department of Public Health grant 340B9930002 and Biornorphics (to S.A.); the Fulbight Foundation (to S.Z.); and the Swedish Foundation for International Cooperation in Research and Higher Education (to M.J.G.). All experimentation involving animals was in accordance with Dana-Farber institutional guidelines. We thank T. W. Mak for CDA44-/- mice; B. J. Rollins for *L. monocytogenes* strain 1778; S. Hikita and D. Block for technical assistance; and A. Angel, K. Mackay, and L. Lagasse for assistance in the preparation of the manuscript.

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STRESS RESPONSE GENES - THE GENES THAT MAKE CANCER METASTASIZE

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Abstract

Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The topology of cancer spread is mediated by a set of developmentally non-essential genes which are physiologically involved in stress responses, inflammation, wound healing, and neovascularization. Function of these gene products is extensively modified posttranscriptionally. In cancer, metastasis genes are dysregulated on the levels of expression or splicing. These genes constitute a unique group of cancer related biomolecules.

Key words

Cancer, metastasis, stress response, posttranslational modification, knockout mouse
What are the traits that make a killer? This question has not only intrigued fans of detective stories it is also most prominent on the minds of cancer researchers. Here the characteristics of the killer are dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The division of normal cells is tightly controlled by dependence on checkpoints which are pauses during the cell cycle where the fidelity of DNA replication and chromosome segregation are monitored. It is regulated by proto-oncogenes, incorporating genes for growth factors, their receptors, and associated intracellular signal transduction molecules. Antagonistic to oncogenes are tumor suppressor genes which normally provide the brakes on cell proliferation. In contrast, cancer is independent of these control mechanisms. Even with defective growth control, however, a cell could never form a tumor of substantial size because, unless it was a germline cell, it would be subject to replicative senescence, an aging process that proceeds with the number of cell divisions and in extreme cases may lead to a state of crisis. A unique role in overcoming replicative senescence is played by the enzyme telomerase which is expressed in virtually all tumor cells but is absent from most normal cells. It prevents telomere shortening with increased number of cell divisions which would eventually cause genomic instability. Mutator genes which encode DNA repair enzymes might be more accurately referred to as meta-oncogenes because their defects give rise to mutations in oncogenes and tumor suppressor genes. Finally, most cells, with the exception of blood and immune cells, grow anchored in their microenvironment whereas cancer cells of particular tissue origin metastasize to specific target organs. The ability of cancer to disseminate throughout the
body also sets it apart from benign tumors, however, the classical cancer genes conspicuously do not account for metastasis formation and current paradigms of cancer have not yet incorporated metastasis genes as a unique group of genes that contributes to the malignant phenotype.

The gene products of stress responses mediate metastasis formation. Lessons from knockout mice.

The topology of metastasis formation is mediated by the potpourri of homing receptors on the tumor cell surface and their ligands and is widely believed to have its physiologic correlate in morphogenesis during embryonic development. This would imply that the deficiency of individual metastasis genes should cause defective formation of the relevant target organ. Unexpectedly, knockout mice in which individual genes known to participate in tumor spread were disrupted turned out to be fertile and developmentally normal (Table 1). This raises the question: What is the physiologic process that has gone astray in cancer dissemination? Despite their diversity, metastasis-associated gene products have several features in common. They comprise a set of genes which physiologically mediate stress responses, including inflammation, wound healing, and neovascularization. Consistently, the defects observed in the relevant gene targeted mice are impairments in these areas. This insight resolves some of the paradoxes of metastasis research. In contrast to morphogenesis, invasiveness and tissue damage are in keeping with the normal functions of host defenses that are executed by macrophages and lymphocytes in stress situations. Homing to and expansion in the lymphoid system, typically the first target in metastatic spread, corroborate the notion that cancer metastasis is based on mechanisms normally
employed by immunocytes¹. Differentiation of immune cells proceeds in the context of their tissue of residence so that lymphocytes from Peyer's patches are distinct from cutaneous lymphocytes and Kupffer cells are distinct from alveolar macrophages. Recognition of topology is encoded in the surface molecules of immune cells and organ preference by cancer may be derived from this principle.

Biologic activity of metastasis mediating gene products is extensively regulated by posttranscriptional mechanisms. Collagenases are typically secreted as precursors whose activation requires proteolytic cleavage, collagenase type IV becomes active after cleavage by stromelysin while prostromelysin and interstitial procollagenase are activated by plasmin. Ligands for homing receptors often contain multiple domains. The heparin-binding amino-terminus of thrombospondin stimulates chemotaxis while the carboxy-terminus mediates haptotaxis in an RGD inhibitable fashion. Comparably, a prerequisite for the interaction of the N-terminal osteopontin domain with integrin receptors is phosphorylation of the cytokine while the C-terminal domain engages variant CD44 by protein-protein interaction. The gene for the homing receptor CD44 contains 10 variant exons that can be spliced into the extracellular domain and determine its engagement of various ligands. Differential effects on binding to extracellular matrix and hyaluronate also depend on the glycosylation and sulfation status of CD44. Posttranscriptional modification of function of these molecules may be beneficial in two ways. Activation by mechanisms like proteolytic cleavage or phosphorylation can be accomplished quickly in stress situations; some of the precursor molecules are widely expressed and can acutely be converted at a site of damage. Also, diversity in structure may encode organ specificity in
homing and metastasis formation (a zip code of sorts). In clinical diagnosis, tumors that grow locally invasive but do not form distal metastases, including cases of basalioma, glioblastoma, chondrosarcoma, and myelomonocytic leukemia, are often referred to as semi-malignant. The molecular mechanisms of local invasion, however, are distinct from conventional forms of cancer only insofar as their target tissues are identical to the tissues of origin.

In conclusion, the topology of cancer spread is regulated by a set of developmentally non-essential genes that physiologically mediate inflammation, wound healing and neovascularization. The function of their products is extensively regulated posttranscriptionally. The entity of these genes encodes the repertoire of stress responses which are predominantly executed by macrophages and lymphocytes. Metastasis-associated gene products therefore constitute a unique and essential group of cancer related biomolecules whose functions are distinct from those of growth control or senescence genes.

Regulation and dysregulation of metastasis genes.

Like yin and yang, phenomena in biology typically have a counter-balance. This holds true for the regulation of cell dissemination. While tumor suppressor genes inhibit cell cycle progression and serve as antagonists for oncogenes, the genes that mediate metastatic spread are balanced by metastasis suppressor genes. The derived gene products typically are adhesion molecules that procure cell anchorage and inhibit migration. Expression of L-CAM is inversely correlated with the metastatic potential of various tumor cell lines. Loss of cadherin expression in squamous cell carcinomas of the head and neck, prostate cancer, and cancers of the female
reproductive tract is associated with poor differentiation and high invasiveness. E-cadherin can prevent the invasive phenotype in T-lymphoma cells. Proteinases also have their antagonists. Tissue inhibitors of metalloproteinases negatively regulate invasion. Their overexpression reduces metastatic potential whereas antisense RNA enhances the malignant phenotype.

It could be argued that metastasis-associated genes are not, in strict terms, cancer genes because mutations in them have not been linked to the risk of contracting cancer. While it is true that these genes have not yet been observed to be mutated in malignancies like the classical oncogenes (frequently through point mutations, deletions, frame shifts, or translocations) they are subject to dysregulation. A case in point is the expression of ICAM-1 on melanoma cells which is an indicator of poor prognosis. Similarly, the homing receptor CD44 is often expressed on cancer cells but not at all on their benign precursors. Alternatively, cancer cells may display splice variants of this receptor which are not detected on their non-transformed counterparts. Therefore, aberration of genes for cancer spread occurs frequently on the levels of transcription or splicing. Without this dysregulation of gene expression tumors could not become malignant.

**Metastasis genes and classical cancer genes. The big picture.**

Even though uncontrolled growth does not inevitably lead to metastatic spread, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection between mutations of oncogenes or tumor suppressor genes and the expression of genes that mediate tumor dissemination. The molecular basis for this connection is currently largely unknown. Expression of metastasis-specific splice variants of CD44 and the oncogene ras
are connected in an autocatalytic mode in which *ras* induces promoter activity for CD44 through an AP-1 binding site while transfection of CD44v enhances the expression of *ras*. This mutual induction may contribute to the perpetuation of cell division and spreading which are characteristic of malignancy. Motility-associated cytokines, including type IV collagenases and osteopontin, can also be induced by *ras* and similar relationships may apply for other oncogenes, including v-*mos*, v-*raf*, v-*fes* and v-*src*².

Recent research has identified the genes that underlie the three phenotypic characteristics of cancer and has allowed a distinction between malignant and benign tumors on the molecular level. Only tumors in which the dysregulation of growth is associated with expression of genes whose products mediate dissemination become malignant. This attributes a central role in carcinogenesis to metastasis genes and metastasis suppressor genes. The definition of molecules that are rarely expressed in the healthy adult organism has given rise to the potential emergence of new drug targets. Among them are telomerase, structurally altered oncogene products such as fusion proteins or mutants, and also some of the stress response molecules that mediate metastasis formation. Prominently, blocking the integrin ανβ3, which is essential for tumor angiogenesis, has been successful in several experimental systems³⁴. Likewise, splice variants of CD44 that mediate dissemination of multiple cancers and are physiologically expressed on immune cells only after antigenic challenge have been targeted in experimental therapy with promising results⁵⁷. Such progress provides the opportunity for a more successful broad attack on the cancer epidemic. As the profile of the killer becomes more refined the prospect for its containment improves.
Acknowledgements

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References


Table 1: Genes that mediate cancer spread are developmentally non-essential. Cancer dissemination is induced by a group of homing receptors, their ligands and proteinases in conjunction with their associated signal transduction molecules. These gene products do not play a critical role in organ development or fertility but are necessary for stress responses. Knockout mice have been generated for multiple metastasis associated genes and uniformly show these characteristics. Various integrins have also been linked to metastasis formation but most integrin gene knockouts display developmental defects. This may be due to the loss of multiple receptors after deletion of individual integrin genes. Furthermore, some intergin gene products serve dual roles in stress responses and development. (DTH = delayed type hypersensitivity, MMP = matrix metalloproteinase, uPAR = receptor for urokinase-type plasminogen activator)

Figure 1: CD44 is essential for metastasis formation by osteosarcoma. C57BL/6 mice with the tm1 point mutation of the p53 gene are susceptible to osteosarcomas which disseminate to liver and lungs. Shown here are a primary tumor (A) and a liver metastasis (B). After disruption of both alleles of the CD44 gene metastatic spread is almost completely abrogated while incidence and growth rate of osteosarcomas are unaffected (Weber et al., manuscript in preparation).
<table>
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Molecular Mechanisms of Tumor Dissemination in Primary and Metastatic Brain Cancers

Running Title: Molecular Mechanisms of Metastasis Formation

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Abstract

Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. Tumor dissemination distinguishes malignant from benign neoplasms and is mediated by homing receptors, their ligands, and proteinases. The homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases. Its engagement by osteopontin physiologically induces macrophage chemotaxis, a mechanism that may be utilized by metastatic brain tumors in the process of dissemination. In host defense, osteopontin and its receptors, CD44 and integrin αvβ3, play key roles in mediating delayed type hypersensitivity responses by activating macrophages to induce Th1 cytokines while inhibiting Th2 cytokines. Other metastasis associated gene products similarly contribute to host defenses. Hence, cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization. Function of the relevant gene products is extensively modified post-transcriptionally and their dysregulation in cancer occurs on the levels of expression and splicing. Consistent patterns of organ preference by malignancies of particular tissue origin suggest a necessary connection between loss of growth control and senescence genes and expression of genes mediating the dissemination of tumor cells.

Key words: Invasion, homing receptors, cytokines, proteinases, stress response
Molecular Characteristics of Cancer

The most prominent feature of malignancy is dysregulated cell cycle progression. Division of cancer cells leads to formation of more cancer cells indicating that the characteristics of transformation originate in genetic changes. The underlying defects causing uncontrolled proliferation are gain of function mutations in oncogenes or loss of function mutations in tumor suppressor genes. However, most somatic cells, with few exceptions such as stem cells, die after a finite number of cell divisions, a phenomenon described as senescence. Replicative senescence begins after fertilization and is genetically dominantly controlled. For cancer to occur, there must be a loss of function in senescence genes or a gain of function in telomerase to give rise to a largely unlimited number of cell divisions. Finally, cancer is distinguished from benign tumors by its faculty to generate metastases. In contrast to earlier models, metastasis formation is a process of active cell migration and invasion rather than the passive dyslocation of cells in the blood or lymph flow. Whether a neoplasm metastasizes and to which target organs is determined by motility associated molecules expressed by the tumor cells.

Invasiveness of Brain Tumors

The brain is unique as a target organ for metastatic growth because it is surrounded by the blood-brain barrier and it lacks lymphatic drainage. Nevertheless, certain malignancies display a preference for dissemination to the central nervous system. Brain metastases from colon and breast cancers are often single whereas melanoma and lung cancer have a greater tendency to produce multiple colonies. At autopsy, up to 80% of melanoma patients have CNS lesions (Sugarbaker
Invasion of brain cancer cells typically proceeds along anatomic structures that are rich in extracellular matrix proteins, including basement membranes of blood vessels and the glial limitans externa (Giese et al. 1996) and has been attributed to specific motility-associated receptors, their ligands and proteinases (Goldbrunner et al. 1999) (Table 1). Specifically, the homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases (Nagasaka et al. 1995; Kuppner et al. 1992; Radotra et al. 1994). Its ligand osteopontin has also been described to be secreted by malignant gliomas (Saitoh et al. 1995; Gladson 1999; Tucker et al. 1998).

**The Physiologic Roles of Metastasis Genes**

To understand the process of metastasis formation we have studied the physiologic importance of the relevant gene products. We have investigated the cytokine osteopontin and its receptor CD44 (Weber et al. 1996; Weber/Cantor 1996; Weber et al. 1997). The engagement of CD44 by osteopontin induces macrophage chemotaxis, a process that may be utilized by metastatic brain tumors in the process of dissemination (Weber et al. 1996). Gene-targeted mice deficient in osteopontin or CD44 are fertile and developmentally normal, a trait that is shared by other knockouts for genes that are believed to be important in metastatic spread. Several observations implied that osteopontin may act as a stress response gene: 1) the osteopontin promoter contains an acute phase responsive element (Kimbro et al. 1995) and a phorbol ester responsive element to which the redox sensitive transcription factors Jun and Fos may bind (Patarca et al. 1993), 2) osteopontin expression by T-lymphocytes, macrophages, and osteoclasts does not occur at rest but is activation dependent and is associated with host resistance (Patarca et al. 1993), 3) osteopontin
exerts anti-oxidant effects and prevents cell damage in response to a large number of noxious influences (Weber et al. 1999), 4) the osteopontin gene knockout results in defective wound healing (Liaw et al. 1998). In host defense, CD44 and its ligand osteopontin play a key role in mediating delayed type hypersensitivity responses by skewing the pattern of cytokines secreted from macrophages to favor the induction of cellular immunity and to suppress humoral immunity. The interaction of osteopontin with its integrin receptor αvβ3 on macrophages stimulates the production of Th1 cytokines while engagement of CD44 by osteopontin concomitantly inhibits the secretion of Th2 cytokines (Ashkar et al. 1999). A classical model of delayed type hypersensitivity is granuloma formation. Foreign body granulomas can be induced by subcutaneous injection of polyvinyl pyrrolidone. After 5 days, control mice display pronounced influx of macrophages and a strong local immune response. In contrast, mice lacking the osteopontin gene due to targeted mutation barely show any immunological reaction to the injection (Figure 1). In contrast, mice lacking the CD44 gene display excessive granuloma formation following challenge (Schmits et al. 1998) which may reflect combined Th1 and Th2 immunity after engagement of integrin receptors by osteopontin in the absence of ligation of CD44.

Preliminary experiments have suggested that CD4^+ T-cells secrete the osteopontin that induces macrophages to selectively promote delayed type immune responses. The observation that macrophages may themselves produce osteopontin after stimulation with lipopolysaccharide raises questions regarding its potential relevance to this process. Macrophage-derived osteopontin is competent for inducing chemotaxis but not delayed type hypersensitivity which may reflect
structural differences from the T-cell secreted molecule. In fact, macrophage osteopontin has lost part of its sequence by alternative splicing (Ashkar and Weber, unpublished observations) which could lead to efficient engagement of CD44 with ensuing chemotaxis but to impaired ligation of integrin receptors. Malignant cells often secrete a form of osteopontin that resembles the macrophage-derived protein in that it may be hypophosphorylated or a splice variant that has a deletion in its N-terminal (integrin binding) portion (Kiefer et al. 1989) and this molecule may contribute to metastatic spread (Weber et al. 1997) by inducing tumor cell migration. Concomitantly, tumor-derived modified osteopontin may ligate CD44 on macrophages without engagement of its integrin receptors (Shanmugam et al. 1997). This leads to suppression of Th2 cytokines while Th1 cytokines cannot be efficiently secreted since other physiologic inducers of Th1 cytokines are substantially less potent. This form of osteopontin action may represent a mechanism of immune evasion.

Tumor dissemination depends on neovascularization. Physiologically, blood vessel formation may be initiated in two settings. The modeling of the cardiovascular system is largely restricted to early development, while in the healthy adult organism, angiogenesis is a rare occurrence that arises predominantly in healing after tissue damage. Morphogenic and stress induced blood vessel generation are mediated by distinct sets of genes. Several pieces of evidence imply a role for osteopontin and its receptors in the latter form of neovascularization. A splice variant of CD44 is involved in endothelial cell proliferation, migration, and angiogenesis (Henke et al. 1996; Trochon et al. 1996). The integrin αvβ3 is of particular importance in angiogenesis due to
its selective expression on growing blood vessels. Antagonists of integrin αβ₃ promote tumor regression by inhibiting neovascularization (Brooks et al. 1994; Arap et al. 1998) and angiogenesis induced by bFGF or by TNFα is also inhibitable by a monoclonal antibody to the integrin αβ₃. Coordinate expression of β₃-integrins and osteopontin by regenerating endothelial cells (Liaw et al. 1995) and during in vitro blood vessel formation (Prols et al. 1998) stimulates migration through cooperative mechanisms involving activation of integrin αβ₃ ligation by thrombin cleavage of osteopontin (Senger et al. 1996).

Conclusions

We conclude, based on our own observations in conjunction with data from the literature, that the topology of cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization and are normally expressed by activated lymphocytes and macrophages (Weber/Ashkar 2000). Function of the relevant gene products is extensively modified post-transcriptionally which allows for quick activation in stress situations and may encode organ specificity. This code for targets in the homing process may cause dissemination to distant organs, such as brain metastases in melanoma or lung cancer, or it may lead to locally invasive growth as is the case in malignant glioma or in chondrosarcoma. In both scenarios, locally destructive growth by malignant glioma and brain metastases from distant primary tumors, the mechanism of invasion is determined by engagement of molecules that are physiologically used by macrophages and
lymphocytes to enter the central nervous system in the context of host defenses, including infection, inflammation, or ischemia.

Indicative of basic mechanisms of homeostasis in human biology all groups of genes involved in malignancies consist of promoting and suppressing components. Loss of function in one group or gain of function in the counterbalancing group may each affect the balance of forces and constitute a predisposing factor for malignant growth. Thus, mutations that enhance the function of oncogenes and mutations that inhibit the function of tumor suppressor genes equally pose a risk for uncontrolled growth of the affected cells and similar relationships hold for senescence genes and metastasis genes and their respective suppressors (Table 2). Furthermore, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection among dysregulated cell cycle control genes (gain of function of oncogenes or loss of function of tumor suppressor genes), suppression of senescence genes, and expression of genes mediating the dissemination of tumor cells. Today, the molecular basis for this connection is largely unknown.
Acknowledgements

This study was supported by National Institutes of Health research grant CA76176 and Department of Defense breast cancer grant DAMD 17-98-1-8060 to GFW and Department of Defense breast cancer grant DAMD 17-99-1-9124 and Department of Public Health grant DPH 34089930002 to SA.
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Legends to Figures and Tables

Table 1: Metastasis-mediating molecules in brain tumors. Specific receptors, ligands (migration inducing cytokines), and proteinases have been associated with the invasive behavior of individual primary and metastatic brain tumors.

Table 2: Genes associated with malignancies. The classical cancer genes (oncogenes and tumor suppressor genes) control cell replication. For cancer to occur, additional functions need to be dysregulated: genes that cause cellular senescence have to be inactivated and expression of gene products that mediate metastasis formation is essential. For cell cycle progression and cell dissemination alike, there is a physiologic balance that may be disturbed by excessive activity of promoters or by diminished function of suppressors. Defects in mutator genes give rise to alterations in other cancer-associated genes putting mutator genes into the position of predisposing factors rather than direct contributors to the malignant phenotype.

Figure 1: Absence of a delayed type hypersensitivity response in mice lacking the osteopontin gene. 250 µg PVP in 500 µl PBS was injected s.c. into the hind limb of C57BL/6 wildtype mice or gene targeted C57BL/6 OPN+/- mice which do not express the osteopontin gene product. After 5 days, histologic analysis of the injection site was performed. 5 µm serial sections were stained with Hematoxilin-Eosin stain. A) injection of PVP into C57BL/6 B) injection of PBS (vehicle control) into C57BL/6 C) injection of PVP into C57BL/6 OPN+/- D) injection of PVP in conjunction with 10 ug purified osteopontin into C57BL/6 OPN+/. Original magnification 200x.
**TABLE 1**

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<td>CD44</td>
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<td>* growth factor receptors</td>
<td>HER-2, erb-B</td>
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<td>* signal transduction molecules associated with growth factor receptors</td>
<td>Akt, Abl, Ras</td>
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<td>* receptors</td>
<td>DCC, PTC</td>
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<td>* signal transduction molecules</td>
<td>p53, Rb, APC</td>
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<td>* cell cycle regulators</td>
<td>p53, Rb, p21, Fos</td>
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<td>* regulators of telomere length</td>
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<td>* proteinases</td>
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<td>* repair of double strand breaks</td>
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**REQUEST**

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

**Box No. I  TITLE OF INVENTION**

**METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE**

**Box No. II  APPLICANT**

<table>
<thead>
<tr>
<th>Name and address:</th>
<th>Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHU &amp; REN'S MEDICAL CENTER CORPORATION</td>
<td>300 Longwood Avenue Boston, Massachusetts 02115 United States of America</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>State (i.e. country) of nationality:</th>
<th>State (i.e. country) of residence:</th>
</tr>
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<tbody>
<tr>
<td>US</td>
<td>US</td>
</tr>
</tbody>
</table>

This person is applicant for the purposes of: all designated States, all designated States except the United States of America, the United States of America only, the States indicated in the Supplemental Box.

**Box III  FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS**

<table>
<thead>
<tr>
<th>Name and address:</th>
<th>Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.</th>
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</thead>
<tbody>
<tr>
<td>DANA-FARBER CANCER INSTITUTE, INC.</td>
<td>44 Binney Street Boston, Massachusetts 02115 United States of America</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>State (i.e. country) of nationality:</th>
<th>State (i.e. country) of residence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>US</td>
</tr>
</tbody>
</table>

This person is: applicant only, inventor only (If this check-box is marked, do not fill in below).

**Box No. IV  AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: agent.

<table>
<thead>
<tr>
<th>Name and address:</th>
<th>Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HANLEY, Elizabeth A.</td>
<td>Lahive &amp; Cockfield, LLP 28 State Street Boston, Massachusetts 02109 United States of America</td>
</tr>
</tbody>
</table>

Telephone No. (617) 227-7400 Facsimile No. (617) 742-4214 Teleprinter No.

Mark this check-box where no agent or common representative is has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

See Notes to the request form
<table>
<thead>
<tr>
<th>Name and address:</th>
<th>State (i.e. country) of nationality:</th>
<th>State (i.e. country) of residence:</th>
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<tr>
<td>ASHKAR, Samy</td>
<td>LB</td>
<td>US</td>
</tr>
<tr>
<td>12 Stoneholm St., Apt. 606</td>
<td>□ all designated States</td>
<td>□ all designated States except the United States of America</td>
</tr>
<tr>
<td>Boston, Massachusetts 02115</td>
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<td>□ the United States of America only</td>
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<tr>
<td>United States of America</td>
<td></td>
<td>□ the States indicated in the Supplemental Box</td>
</tr>
</tbody>
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This person is:  
- □ applicant only  
- X applicant and inventor  
- □ inventor only (If this check-box is marked, do not fill in below.)

<table>
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<tr>
<th>Name and address:</th>
<th>State (i.e. country) of nationality:</th>
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<tr>
<td>WEBER, Georg</td>
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<tr>
<td>163 Bellingham Rd</td>
<td>□ all designated States</td>
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<tr>
<td>Chestnut Hill, MA 02647</td>
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</table>

This person is:  
- □ applicant only  
- X applicant and inventor  
- □ inventor only (If this check-box is marked, do not fill in below.)

<table>
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<td>GLIMCHER, Melvyn</td>
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<td>□ the United States of America only</td>
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<td>□ the States indicated in the Supplemental Box</td>
</tr>
</tbody>
</table>

This person is:  
- □ applicant only  
- X applicant and inventor  
- □ inventor only (If this check-box is marked, do not fill in below.)

<table>
<thead>
<tr>
<th>Name and address:</th>
<th>State (i.e. country) of nationality:</th>
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</tbody>
</table>

This person is:  
- □ applicant only  
- X applicant and inventor  
- □ inventor only (If this check-box is marked, do not fill in below.)

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.
The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

**Regional Patent**

- AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- EP European Patent: AT Austria, BE Belgium, CH Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line).

**National Patent**

If other kind of protection or treatment desired, specify on dotted line:

- AE United Arab Emirates
- AL Albania
- AM Armenia
- AT Austria
- AU Australia
- AZ Azerbaijan
- BA Bosnia and Herzegovina
- BB Barbados
- BG Bulgaria
- BR Brazil
- BY Belarus
- CA Canada
- CH Switzerland and Liechtenstein
- CN China
- CR Costa Rica
- CU Cuba
- CZ Czech Republic
- DE Germany
- DK Denmark
- DM Dominica
- DZ Algeria
- EE Estonia
- ES Spain
- FI Finland
- GB United Kingdom
- GD Grenada
- GE Georgia
- GH Ghana
- GM Gambia
- HR Croatia
- HU Hungary
- ID Indonesia
- IL Israel
- IN India
- IS Iceland
- JP Japan
- KE Kenya
- KG Kyrgyzstan
- KP Democratic People's Republic of Korea
- KR Republic of Korea
- KZ Kazakhstan
- LC Saint Lucia
- LK Sri Lanka
- LR Liberia
- LS Lesotho
- LT Lithuania
- LU Luxembourg
- LV Latvia
- MA Morocco
- MD Republic of Moldova
- MG Madagascar
- MK The former Yugoslavia Republic of Macedonia
- MN Mongolia
- MW Malawi
- MX Mexico
- NO Norway
- NZ New Zealand
- PL Poland
- PT Portugal
- RO Romania
- RU Russian Federation
- SD Sudan
- SE Sweden
- SG Singapore
- SI Slovenia
- SK Slovakia
- SL Sierra Leone
- TJ Tajikistan
- TM Turkmenistan
- TR Turkey
- TT Trinidad and Tobago
- TZ United Republic of Tanzania
- UA Ukraine
- UG Uganda
- US United States of America (as a CIP)
- UZ Uzbekistan
- VN Viet Nam
- YU Yugoslavia
- ZA South Africa
- ZW Zimbabwe

**Precautionary Designation Statement:** In addition to the designations made above, the applicant also makes under Rule 4.9(h) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)
Continuation of Box IV

Agent:

LIEPMANN, W. Hugo
COCKFIELD, James E.
SMURZYNSKI, Thomas V.
LOREN, Ralph A.
DECONTI, Jr., Giulio A.
LYNCH, Jeremiah
HAMMITTE, Ann Lamport
MANDRAGOURAS, Amy E.
LAURENTANO, Anthony A.
REMLILLARD, Jane E.
CANNING, Kevin J.
LANE, Jr., David A.
KARA, Catherine J.
LAURO, Peter C.
DIGIORGIO, Jeanne M.
WILLIAMS, Megan E.
TRIANO III, Nicholas P.
WELCH, John L.
DOUROS, Timothy J.
SMITH, DeAnn F.
DeVAUL, William D.
RIKKERS, David J.
KIM, Chi Suk

The above attorneys and agents are members of the firm of Lahive & Cockfield, LLP. Address, telephone number, facsimile number and teleprinter number of all are indicated in Box IV.

Continuation of Box V.

Continuation-in-part
United States of America

Application No.: 60/129,772
Filed 15 April 1999 (15.04.99)
Box No. VI  PRIORITY CLAIM

<table>
<thead>
<tr>
<th>Filing date of earlier application (day/month/year)</th>
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<th>Regional application: regional Office</th>
<th>International application: receiving Office</th>
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<tr>
<td>item (1) 15 April 1999 (15.04.99)</td>
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<td>item (2)</td>
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The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): (1)

*Where the earlier application is an ARIPRO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII  INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year) Number Country or regional Office

ISA/EP

Box No. VIII  CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

- Request: 5
- Description (excluding sequence listing part): 94
- Claims: 9
- Abstract: 1
- Drawings: 7
- Sequence listing part of description: 9

Total number of sheets: 109

This international application is accompanied by the item(s) marked below:

1.  
2.  
3.  
4.  
5.  
6.  
7.  
8.  
9.  
10.  

Figure of the drawings which should accompany the abstract: NONE

Language of filing of the international application: ENGLISH

Box No. IX  SIGNATURE OF APPLICANT OR AGENT

For CHILDREN'S MEDICAL CENTER CORPORATION, et al.

Catherine J. Kara

For receiving Office use only

1. Date of actual receipt of the purported international application:

2. Drawings:

   - received:
   - not received:

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

See Notes to the request form.
**PCT FEE CALCULATION SHEET**

**Annex to the Request**

<table>
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<th>No.</th>
<th>Description</th>
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<th>Fee</th>
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<td>International search to be carried out by</td>
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<tr>
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<td>3.</td>
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<tr>
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<td>427 b1</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)</td>
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<tr>
<td>4.</td>
<td>FEE FOR PRIORITY DOCUMENT (if applicable)</td>
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<td><strong>TOTAL</strong></td>
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</tbody>
</table>

- The designation fee is not paid at this time.

**MODE OF PAYMENT**

- authorization to charge
- deposit account (see below)
- cheque
- postal money order
- bank draft
- cash
- revenue stamps
- coupons
- other (specify):

**DEPOSIT ACCOUNT AUTHORIZATION** (this mode of payment may not be available at all receiving Offices.)

- THE RO/US is hereby authorized to charge the total fees indicated above to my deposit account.
- is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.
- is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

**Deposit Account Number**

**Date (day/month/year)**

**Signature**

---

Form PCT/RO/101 (Annex) (January 2000)