mda-7/IL-24, novel anticancer cytokine: Focus on bystander antitumor, radiosensitization and antiangiogenic properties and overview of the phase I clinical experience (Review)

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Abstract. Subtraction hybridization applied to a 'differentiation therapy' model of cancer employing human melanoma cells resulted in the cloning of melanoma differentiation associated gene-7/interleukin-24 (*mda*-7/IL-24). Initial studies confirm an inverse correlation between *mda*-7 expression and melanoma development and progression. Forced expression of *mda*-7 by means of a plasmid or via a replication incompetent adenovirus (Ad.*mda*-7) promotes growth suppression and induces apoptosis in a broad array of human cancers. In contrast, *mda*-7 does not induce growth suppressive or toxic effects in normal cells. Based on structure (containing an IL-10 signature motif), secretion by cells (including subsets of T-cells) and location on chromosome 1q (in an area containing IL-10-family genes), *mda*-7 has now been renamed *mda*-7/IL-24. Studies by several laboratories have uncovered many of *mda*-7/IL-24's unique properties, including cancer-specific apoptosis-induction, cell cycle regulation, an ability to inhibit angiogenesis, potent 'bystander antitumor activity' and a capacity to enhance the sensitivity of tumor cells to radiation, chemo-

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Abbreviations: Ad, adenovirus; APC, adenomatosus polyposis coli gene; bFGF, basic fibroblast growth factor; CLL, chronic lymphocytic leukemia; DOTAP:Chol, 1,2-dioleoyl-3-(trimethylammonio) propane: Cholesterol; COX-2, cyclooxygenase-2; CR, complete response; ECD, endothelial cell differentiation; ER, endoplasmic reticulum; FADD, Fas-associated death domain; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FISP, IL-4-induced secreted protein; FITC, fluorescein isothiocyanate; *GADD* genes, growth arrest and DNA damage inducible genes; GSK-3ß, glycogen synthase kinase-3ß; GM-CSF, granulocyte macrophage colony-stimulating factor; HUVEC, human umbilical vein endothelial cells; IKK, IκBα kinase; IR, ionizing radiation; JAK-STAT, janus kinase signal transduser and activator of transcription; *mda*-7, melanoma differentiation associated gene-7; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblasts; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear leukocytes; *PEG-3*, progression elevated gene-3; PHA, phytohemagglutinin; PI3K, phosphatidylinositol 3-kinase; PKR, double-stranded RNA-dependent protein kinase; PR, partial response; ROS, reactive oxygen species; SAE, serious adverse effects; SCCHN, squamous cell carcinoma of the head and neck; SD, stable disease; SMS, smooth muscle cell; TCF/LEF, T-cell factor/lymphoid enhancer factor; TGF-ß, transforming growth factor-ß; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; UPR, unfolded protein response; VEGF, vascular endothelial growth factor; vp, viral particles

therapy and monoclonal antibody therapy. Moreover, based on its profound cancer tropism, substantiated by *in vivo* human xenograft studies in nude mice, *mda*-7/IL-24 (administered as Ad.*mda*-7) was evaluated in a phase I clinical trial in patients with melanomas and solid cancers. These studies document that *mda*-7/IL-24 is well tolerated and demonstrates evidence of significant clinical activity. In these contexts, *mda*-7/IL-24 represents a unique cytokine gene with potential for therapy of human cancers. The present review focuses on three unique properties of *mda*-7/IL-24, namely its potent 'bystander anti-tumor activity', ability to sensitize tumor cells to radiation, and its antiangiogenesis properties. Additionally, an overview of the phase I clinical trial is provided. These studies affirm that *mda*-7/IL-24 has promise for the management of diverse cancers.

Contents

1. Introduction

A novel melanoma differentiation associated (*mda*) gene, *mda*-7, was identified with a subtraction hybridization protocol (1) using temporally-spaced cDNA libraries isolated from terminally differentiated human melanoma cells treated with ß-interferon and the protein kinase C activator mezerein (2). The *mda*-7 cDNA encodes a 24-kDa protein, which contains an interleukin (IL)-10 signature motif at amino acids 101-121 (SDAESCYLVHTLLEFYLKTVF) shared by other members of the IL-10 family of cytokines (3,4). Based on sequence homology, chromosomal localization, and its functional properties, the *mda*-7 gene is now classified as a member of the IL-10 family of cytokines and named IL-24 (4-8). The MDA-7/IL-24 protein contains a consensus signal sequence and a proteolytic cleavage site permitting secretion from cells. Expression of MDA-7/IL-24 protein in normal cells is restricted to cells of the immune system and melanocytes (5,7,9). MDA-7/IL-24 expression is decreased during pathologic progression of melanocytes to melanomas as demonstrated by both RT-PCR (2) and immunohistochemistry (10). This correlation between the loss of gene expression and tumor invasion suggests that *mda*-7/IL-24 might function as a tumor suppressor (2,11,12). Further studies *in vitro* indicated that the functional antitumor attributes of *mda*-7/IL-24 were dependent on MDA-7/IL-24 expression level (13). When present at low physiological levels, *mda*-7/IL-24 exhibited specific immunostimulatory properties (see details below). However, when this gene was overexpressed in diverse cancer cells *in vitro* by transfection with a *mda*-7/IL-24 cDNA or by adenoviral infection with a recombinant replication incompetent adenovirus, Ad.*mda*-7, it displayed potent tumor killing and apoptosis-inducing properties (reviewed in refs. 4,6,8,14-20).

Infection with Ad.*mda*-7 results in apoptosis induction and cell death in a wide variety of solid tumors including melanoma, malignant glioma, fibrosarcoma and carcinomas of the breast, cervix, colorectum, liver, lung, ovary and prostate. The tumor suppressing ability of *mda*-7/IL-24 is now well established and is independent of the status of other tumor suppressor genes, such as *p53* and *Rb*, or apoptosis regulating genes, such as *bax* or *caspases*, in tumor cells (12,21-25). A provocative discovery was that overexpression of *mda*-7/IL-24 in normal cells, such as normal human epithelial cells, melanocytes, astrocytes or fibroblasts, did not affect their growth or viability. This *in vitro* tumor-selective cytotoxicity was confirmed by *in vivo* experiments in animal models containing human breast, lung and colorectal carcinomas and in glioma xenografts (23-26). Based on the results obtained using *in vitro* studies and in *in vivo* preclinical animal modeling, *mda*-7/IL-24 emerged as a promising new and potentially widely applicable antitumor therapeutic agent, possessing direct tumor growth inhibitory effects as well as 'bystander antitumor' properties. Findings from a completed phase I dose-escalation trial on 22 advanced cancer patients who received intratumoral injection of a non-replicating adenovirus vector carrying the *mda*-7/IL-24 transgene (INGN 241) were recently reviewed (6,17,18,27-29). These results confirm that the clinical activities of INGN 241 (Ad.*mda*-7) include apoptotic and antitumor activities and systemic immune activation and can generate both direct tumor growth inhibition and 'bystander antitumor' effects.

2. Mechanisms of antiapoptotic properties of *mda*-7/IL-24

IL-20/IL-22 receptor independence. Within the IL-10 family, IL-19, IL-20 and IL-24 exhibit substantial sharing of receptor complexes; all three are capable of signaling through IL-20R1/IL-20R2, and both IL-20 and IL-24 can also use IL-22R1/IL-20R2 (30). Nevertheless, the biological activities of these three cytokines appear quite distinct; since only *mda*-7/IL-24 induces tumor-specific apoptosis, and this effect can be receptor-independent (31). The MDA-7/IL-24 protein binds to IL-20 and IL-22 receptor complexes resulting in JAK/STAT activation (30,32,33). However, treatment with tyrosine kinase-specific inhibitors (Genistein and AG18) or a JAK-selective inhibitor (AG490) does not alter Ad.*mda*-7-induced apoptosis in diverse cancer cell lines (31). In addition, there is no correlation between the pattern of expression of IL-20R1, IL-20R2, and IL-22R mRNA and susceptibility to Ad.*mda*-7-induced cell death in different cell lines. Therefore, signaling events leading to Ad.*mda*-7-induced apoptosis might be tyrosine kinase independent and can be distinguished from *mda*-7/IL-24 cytokine function-related properties mediated by the IL-20/IL-22 receptor complexes that require JAK/STAT kinase activity (31,34,35).

Mitochondrial pathway and reactive oxygen species (ROS) production. *In vitro* studies were performed to address the mechanism(s) by which overexpression of *mda*-7/IL-24

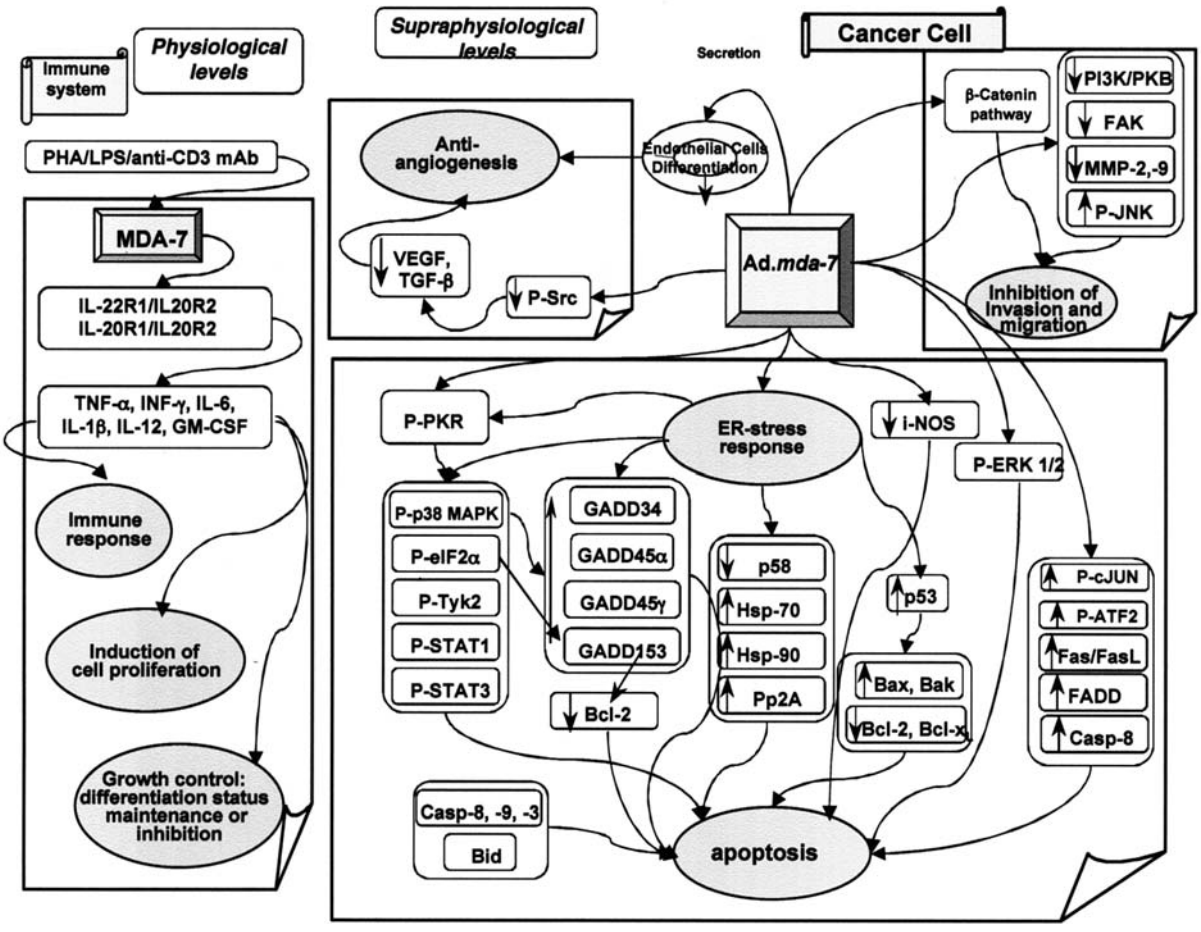

Figure 1. Model of *mda*-7/IL-24-induced apoptosis in cancer cells. Outline of proposed physiologic (left) and ectopic (right) effects of *mda*-7/IL-24 on normal and cancer cells. When normally or ectopically overexpressed, MDA-7/IL-24 localizes to the ER/Golgi compartments, whether or not the protein contains a secretory signal. Accumulation of MDA-7/IL-24 protein in this compartment triggers apoptosis that could involve induction of ER stress and/or reactive oxygen species in mitochondria. Secreted MDA-7/IL-24 protein employs the IL-20/IL-22 receptors to activate signal transduction pathways and/or potentially enter cancer cells and activate proapoptotic pathways by localization and accumulation in the ER/Golgi compartment and/or by inducing mitochondrial dysfunction. A combination of pathways triggered by *mda*-7/IL-24 results in transformed cell-specific apoptosis. Modified from Lebedeva *et al* (17).

induces death selectively in cancer cells. Despite these efforts, the precise pathways of *mda*-7/IL-24-induced apoptosis remain to be defined. Evidences from earlier studies suggested an involvement of the intrinsic/mitochondrial pathway of apoptosis. Transfection of breast carcinoma cells with an *mda*-7/IL-24 cDNA led to proapoptotic Bax up-regulation and subsequent apoptosis induction (25,36). The onset of apoptosis was blocked by antiapoptotic Bcl-2 overexpression. Further studies in melanoma (12), glioblastoma multiforme (26,37,38), renal carcinoma (39) and prostate carcinomas (21,40,41) confirmed the involvement of the Bcl-family of proteins in *mda*-7/IL-24-induced apoptosis. In most tumor cell contexts, overexpression of MDA-7/IL-24 protein leads to the significant down-regulation of antiapoptotic (Bcl-2 and Bcl-x_L) and/or to the up-regulation of proapoptotic (Bax and Bak) members of the Bcl-family. It should be noted that Ad.*mda*-7 can induce apoptosis in *bax*-null DU-145 prostate cancer cells (21); therefore, apoptosis can be mediated by a *bax*-independent pathway. Moreover, overexpression of anti-apoptotic Bcl-family members differentially protects prostate cancer cells from the *mda*-7-induced apoptosis (21). Bcl-x_L overexpression prevents Ad.*mda*-7-induced apoptosis in DU-145 and PC-3 prostate carcinoma cells, while Bcl-2 over-expression protects LNCaP cells from *mda*-7/IL-24-induced apoptosis. The reasons for this disparity are not understood and are currently being investigated.

Detailed studies in prostate cancer cells identify reactive oxygen species (ROS) as an active component in *mda*-7/IL-24-induced apoptosis (Fig. 1) (39,42,43). When overexpressed inside a cancer cell MDA-7/IL-24 protein directly or indirectly affects mitochondria, causing mitochondrial dysfunction and the inner mitochondria membrane potential to drop thereby leading to ROS production and to apoptosis induction. All of the changes described above are absent after Ad.*mda*-7 infection of normal prostate epithelial cells. Antioxidants (*N*-acetyl-$_L$-cysteine and Tiron) and inhibitors of mitochondrial permeability transition (cyclosporine A and bongkrekic acid) inhibit Ad.*mda*-7-induced mitochondrial dysfunction and apoptosis in prostate cancer cells. Conversely, agents augmenting ROS production (arsenic trioxide, NSC656240, and PK11195) facilitate Ad.*mda*-7-induced apoptosis. Ectopic expression of *Bcl*-2 and *Bcl*-x_L inhibits mitochondrial changes, ROS production, and apoptosis providing additional support for an association between mitochondrial dysfunction and Ad.*mda*-7 action. These studies present definitive evidence that changes in mitochondrial function and ROS production represent key components associated with selective killing of prostate cancer cells by *mda*-7/IL-24.

The ability of MDA-7/IL-24 to produce ROS may prove extremely important for treatment of pancreatic cancer. Pancreatic cancer cells are resistant to *mda*-7/IL-24-induced apoptosis due to a diminished capacity to convert *mda*-7/IL-24 mRNA into protein (44). This translational block can be reversed by combinational treatment with Ad.*mda*-7/IL-24 and agents that increase the ROS levels within cells, such as arsenic trioxide, N-(4-hydroxyphenyl) retinamide, or dithiophene (42). Induction of apoptosis *in vitro* and suppression of tumorigenesis *in vivo* in nude mice are induced in pancreatic cancers independently of their K-*ras* status upon combinatorial treatment with Ad.*mda*-7/IL-24 and a ROS-inducing agent. ROS inhibitors, including N-acetyl-cysteine and Tyron, block this effect.

ER-stress and its link to mitochondria. In addition to modification of mitochondrial function, *mda*-7/IL-24 has also been shown to kill cancer cells by causing endoplasmic reticulum (ER) stress (45-47). ER stress may be caused by misfolded protein accumulation followed by the activation of a highly conserved unfolded protein response (UPR). These events lead to apoptosis through the induction of growth arrest and DNA-damage-inducible (*GADD*) genes (48,49). The hypothesis of a role for *mda*-7/IL-24-induced ER stress and cancer cell apoptosis is supported by the fact that *mda*-7/IL-24 induces *GADD* genes and further activates p38MAPK in the context of transformed cells (46,50).

Previous studies document that signaling events leading to Ad.*mda*-7-induced transformed cell apoptosis are tyrosine kinase-independent (31). These results suggest that *mda*-7/IL-24 cancer cell-specific activity can occur through mechanisms independent of binding to its currently recognized cognate receptors and might even occur independent of receptor function. An adenovirus vector expressing a non-secreted version of MDA-7/IL-24 protein was generated by deletion of its signal peptide (45). This non-secreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis in prostate carcinoma cell lines and displayed transformed cell specificity and localization of MDA-7/IL-24 in the Golgi/ER compartments. These results indicate that *mda*-7/IL-24-mediated apoptosis can be triggered through a combination of intracellular as well as secretory mechanisms and could occur efficiently in the absence of protein secretion.

Treatment of susceptible prostate cancer cell lines with Ad.*mda*-7 as well as Ad.SP-*mda*-7 induces killing to a comparable extent through ERK1/2-dependent and JAK/STAT-independent pathways (45). The fact that both secreted and non-secreted forms of MDA-7/IL-24 protein have comparable apoptosis-inducing activity was unanticipated, adding an additional level of complexity in understanding how this novel molecule works. Localization of full-length MDA-7/IL-24 protein in the ER/Golgi compartments is consistent with the signal peptide hypothesis (51) and the currently known and predicted secreted cytokine nature of the protein (6,14). Because the signal-peptideless mutant of MDA-7/IL-24 protein does not contain an export signal, it is predicted to remain in the cytosol. We have, however, confirmed through confocal immunofluorescence studies that a significant fraction of this protein is able to enter the ER and Golgi apparatus and that proteins derived from wild-type and mutant viruses appear to have overlapping patterns of localization within the cell (45). It is not possible to rule out cryptic internalization signals that become active in the absence of the actual signal peptide, as the identity of these cryptic sites is currently hypothetical. Western blot analyses performed on protein-derived cytosolic and extracellular fractions of cells infected with both viruses indicated that only full-length MDA-7/IL-24 was processed and secreted. It is also possible that adenovirus infection produced relatively large amounts of protein that even in the absence of a specific targeting sequence possesses the ability to cross membranes and accumulate in the ER/Golgi because of charge and/or tertiary structure. However, because localization of MDA-7/IL-24 is similar in both normal (P69) and cancer (DU-145) cells, differences in cellular localization of this protein can be excluded as a direct mechanism underlying the differential apoptosis-inducing activity of MDA-7/IL-24 toward cancer cells. There are two possible explanations of differential MDA-7/IL-24-induced killing in tumor and normal cells. One is that enhanced sensitivity may be due to the 'activated' or 'destabilized' nature of tumor compared to normal cells, which enhances cell death after the ER-stress response is triggered. The other possibility is that *mda*-7/IL-24 not only induces the classical ER-stress response that favors apoptosis, but it also induces additional specific pathways that cause apoptosis only in transformed cell lines. We demonstrated that *mda*-7/IL-24 localizes to the ER compartment both in normal and cancer cells and is therefore in a position to induce this pathway irrespective of the transformation status of the cell (45). Current data provide some support for both hypotheses, however additional studies are required. *GADD* family gene induction as well as p38 MAPK activation is induced only in transformed and not in normal cells (46,50) indicating that the disparate response might be due to differential activation, either in strength or in duration of the ER-stress response. However, this does not rule out the activation of additional pathways, specifically in cancer cells. In a microarray-based study, *mda*-7/IL-24 was shown to induce expression of ER-stress response genes such as *BiP/GRP78*, *PP2A*, *HSJ1* and *TRA1* in H1299 lung carcinoma cells (52). Nevertheless, with the exception of *BiP/GRP78*, which is selectively up-regulated in cancer cells (46), no comparative data for most of these genes is available contrasting normal and cancer cells.

MDA-7/IL-24 modulates multiple apoptotic signaling pathways. Expanding studies in diverse systems reveal that the signal transduction pathways mediating *mda*-7/IL-24-induced apoptosis are varied and multiple signaling pathways are activated in different tumor cell lines upon Ad.*mda*-7 infection or treatment with recombinant GST-MDA-7 protein (53) (summarized in Fig. 2). Experiments in melanoma cell lines demonstrated that SB203580, a specific inhibitor of p38 MAPK pathway, protects these cells from Ad.*mda*-7-induced apoptosis. Ad.*mda*-7 infection resulted in phosphorylation of p38 MAPK and induction of the *GADD* family of genes in melanoma cells, but not in normal melanocytes (50). In general, overexpression of each *GADD* gene from the family in combination (*GADD34, GADD45α, GADD45β, GADD45γ* and *GADD153*) led to synergistic or cooperative antiproliferative effects (54). Ad.*mda*-7 infection resulted in significant

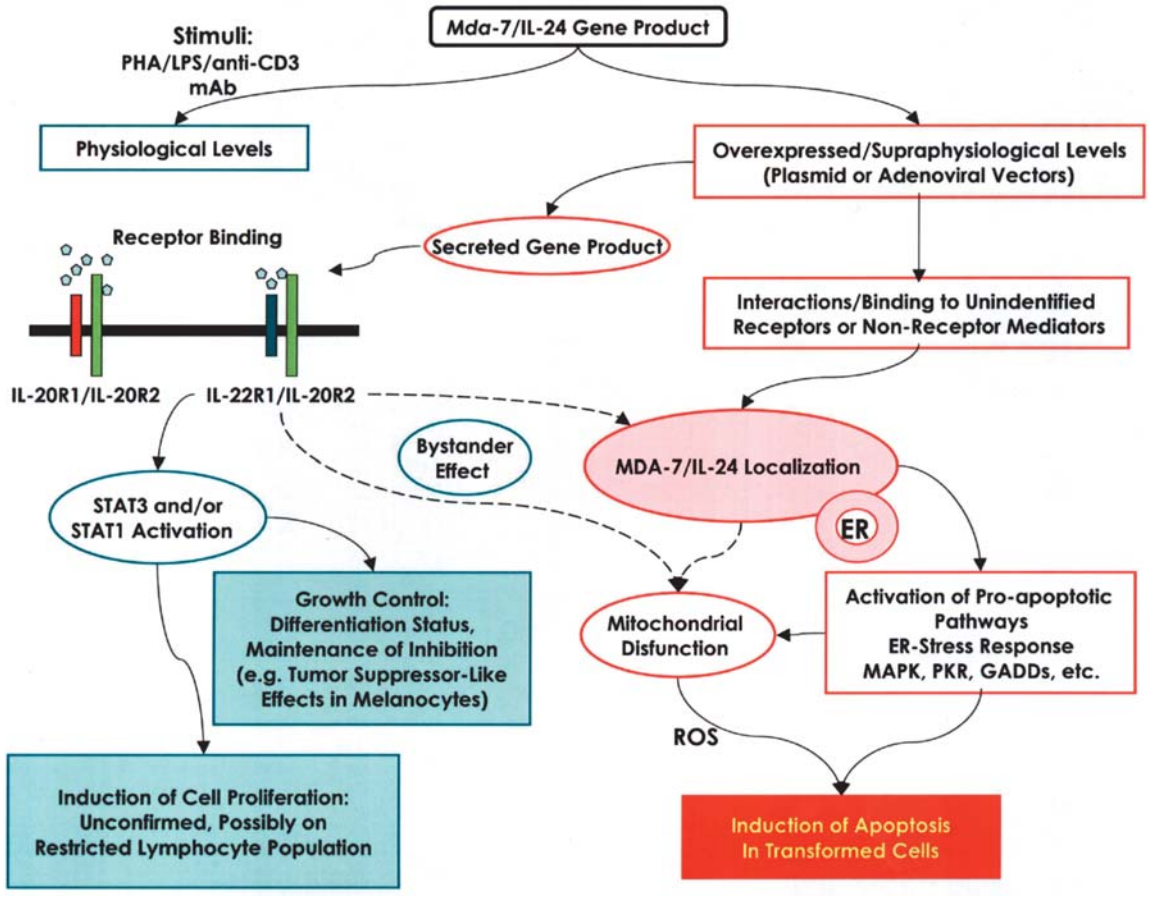

Figure 2. Signaling pathways involved in *mda*-7/IL-24 activity in cancer cells, endothelial cells and immune system cells. Overview of the signaling pathways associated with Ad.*mda*-7 and MDA-7/IL-24 activity in cancer cells and in the immune system. Modified from Sauane *et al* (45).

induction of *GADD153*, *GADD45α* and *GADD34*, and a moderate induction of *GADD45γ* (50). Both inhibition of the p38 MAPK pathway (either pharmacologically with SB203580 or by an adenovirus expressing a dominant negative p38 MAPK) and inhibition of the *GADD* family of genes by an antisense approach rescued melanoma cells from Ad.*mda*-7-induced apoptosis. Activation of the p38 MAPK pathway followed by induction of the *GADD* family of genes also plays a crucial role in Ad.*mda*-7-modulated apoptosis in glioblastoma multiforme (37), prostate cancer (31,41,46), and breast cancer cells (unpublished data).

Paradoxically, p38 MAPK phosphorylation by MDA-7/IL-24 in chronic lymphocytic leukemia (CLL)-B-cells promoted survival of these malignant cells (55). Both *mda*-7/IL-24 mRNA and protein were overexpressed in the CLL B-cells examined, and p38 MAPK, a downstream *mda*-7/IL-24 signaling target, was highly phosphorylated in all CLL cells, but not in normal B-cells. Obviously, phospho-p38 MAPK has no proapoptotic functions in CLL cells and may instead be required for survival, as suggested from these studies. Correspondingly, phosphorylation of p38 MAPK following transfection of CLL cells with *mda*-7/IL-24 promoted CLL cell survival (55). These studies do not exclude the possibility of *mda*-7/IL-24 signaling through a p38 MAPK-independent mechanism; however, their data suggest a competition between *mda*-7/IL-24 and SB203580 (a specific inhibitor of p38 MAPK) for p38 MAPK activation.

In non-small cell lung carcinoma (NSCLC) cells, Ad.*mda*-7 induced apoptosis via up-regulation of double stranded RNA-dependent protein kinase (PKR). Infection with Ad.*mda*-7 led to the phosphorylation of PKR and also its downstream targets eIF2α, Tyk2, Stat1, Stat3, and p38 MAPK (56,57). Ad.*mda*-7 treatment activated caspases 3, 8 and 9 and cleavage of Bid and PARP in NSCLC cells. The activation of PKR appeared to be upstream of caspase activation because pre-treatment with caspase inhibitors failed to prevent PKR phosphorylation. Treatment with a specific serine/threonine kinase inhibitor 2-aminopurine blocked Ad.*mda*-7-induced apoptosis and also activation of PKR and eIF2α (56). A recent study by Chada *et al* (58) reported PKR up-regulation in breast cancer cell lines upon Ad.*mda*-7 infection. Further investigation of the interaction between MDA-7/IL-24 and PKR in NSCLC cells suggested a role for posttranslational regulation of PKR by MDA-7/IL-24 (59). A physical interaction of MDA-7/IL-24 and PKR was confirmed by immunofluorescence and coimmunoprecipitation studies. In both studies (56,59), the authors indicate an inability of Ad.*mda*-7 to induce apoptosis in PKR null (-/-) mouse embryonic fibroblasts (MEF), but not in PKR wild-type MEFs, providing additional proof of PKR involvement in Ad.*mda*-7-modulated apoptosis. Nevertheless, this finding requires further independent confirmation given that Ad.*mda*-7 does not induce apoptosis in a wide array of normal human or rodent cells.

Activation of p38 MAPK following Ad.*mda*-7 infection appears to be a common event linking the PKR and p38 MAPK pathways (50,56,57). It is possible that in melanoma cells p38 MAPK activation is downstream of PKR activation, although in melanoma cells the post-p38 signal transduction changes seem to be more important in Ad.*mda*-7-induced apoptosis. Since eIF2α phosphorylation activates the transcription factor ATF4, which in turn activates *GADD153* (60), there is a significant level of cross talk between the PKR and the p38 MAPK signal transduction pathways. Additional studies are required to identify the upstream molecules within PKR and p38 MAPK pathways involved in Ad.*mda*-7-induced apoptosis.

The c-Jun NH_2-terminal kinase (JNK) pathway is another important signaling pathway involved in *mda*-7/IL-24-induced apoptosis. Infection with Ad.*mda*-7 radiosensitizes malignant glioma (37,61) and prostate cancer cells (40). A combination of Ad.*mda*-7 and γ-irradiation activates JNK in glioma and prostate cancer cells, and treatment with a specific JNK inhibitor, SP600125, prevents apoptosis after the combination treatment (40,61). JNK activation leads to down-regulation of antiapoptotic Bcl-x_L and/or Bcl-2 in glioma and prostate carcinoma cells (38,40) and to the up-regulation of proapoptotic Bax and Bak proteins in prostate carcinoma cells (40). Blocking of JNK phosphorylation with SP600125 abrogates these changes in the levels of Bcl-family proteins. In NSCLC cells, curcumin (dietary pigment that inhibits JNK activation) prevents phosphorylation of c-*jun* and radiosensitization by Ad.*mda*-7 (62).

A potential negative regulation of ß-catenin and PI3K/Akt signaling pathways, which are implicated in cell-cell adhesion, cytoskeletal rearrangements, and membrane trafficking in breast and lung cancer cells, by MDA-7/IL-24 was reported (63). Using microarray analysis, increased protein expression was evident from tumor suppressor genes such as *E-cadherin, APC, GSK-3ß*, and *PTEN* after Ad.*mda*-7 infection. At the same time, expression of proto-oncogenes involved in ß-catenin and PI3K signaling was decreased. Ad.*mda*-7 treatment led to a redistribution of cellular ß-catenin from the nucleus to the plasma membrane. As a result, LEF/TCF transactivation was significantly reduced, and E-cadherin-ß-catenin adhesion complex was up-regulated in a tumor cell-specific manner. Furthermore, Ad.*mda*-7 infection of breast and lung cancer cells down-regulated the expression of PI3K pathway members (p85 PI3K, FAK, ILK-1, Akt, and PLC-γ). Experiments did not indicate if overexpression of ß-catenin or activation of the PI3K pathway protects these cells from Ad.*mda*-7-induced apoptosis. In conclusion, Ad.*mda*-7 appears to negatively regulate both the PI3K and the ß-catenin signaling pathways in breast and lung cancer cells resulting in restoration of apoptosis induction. Moreover, PI3K activation has been implicated in chemoresistance and radioresistance in tumor cells; thus, Ad.*mda*-7 treatment should serve as a chemo- and radiosensitizer. Ad.*mda*-7 regulates multiple members of the ß-catenin and PI3K pathways that are considerably redundant and simultaneously produce antiproliferative, proapoptotic, and antimetastatic phenotypes. Although it is not clear where these changes are initiated, *mda*-7/IL-24 appears to act upstream of PI3K, PLC-γ, and PTEN. It is significant that the ß-catenin and PI3K pathways were not altered by *mda*-7/IL-24 expression in normal HUVEC cells.

Activation of the Fas-FasL signaling pathway occured in the human ovarian cancer cell line MDAH 2774 following infection with Ad.*mda*-7, where Ad.*mda*-7 significantly inhibited cell proliferation and induced apoptosis. Early *mda*-7/IL-24-induced activation of the transcription factors c-Jun and activating transcription factor 2 (ATF2), which stimulated the transcription of an immediate downstream target, the death-inducer Fas ligand (FasL), and its cognate receptor Fas. The activation of NF-κB and induction of Fas-associated factor 1, FADD, and caspase-8 were associated with the activation of Fas-FasL (64). However, another study by Emdad *et al* (65) reported p38 MAPK activation upon adenovirus-mediated *mda*-7 infection in several ovarian cancer cell lines. The level of phosphorylated p38 MAPK correlated with the killing effect of *mda*-7/IL-24 in these cells, and SB203580 (selective p38 MAPK inhibitor) significantly abolished this killing effect. Thus, activation of p38 MAPK presents a more general effect than induction of the Fas-FasL pathway and might be a key element in *mda*-7/IL-24-induced apoptosis in ovarian carcinoma cell lines as well as in other cancer cell lines (46,50,61,65).

3. *mda*-7/IL-24 expression radiosensitizes cancer cells

Radiotherapy is a common option for cancer therapy; however, because of high toxicity at therapeutic doses the efficiency of this treatment is often suboptimal. Ionizing radiation (IR) generates hydroxyl radicals in water that can impact the function of mitochondria in cells, resulting in amplification of the amount of reactive oxygen and nitrogen species (66,67). In studies using human malignant glioma (26,37,38,61) and prostate carcinoma (40), the ability of both IR and *mda*-7/IL-24 to generate ROS was directly linked to the radiosensitizing properties of this gene. Additional studies in ovarian carcinoma (65), NSCLC (23,62) and breast cancer cells (58) confirmed the radiosensitizing ability of *mda*-7/IL-24 gene. Studies *in vitro* highlighted the importance of JNK activation as a mediator of radiosensitization effect and its p53 independence (6,26,37,38,40,61,62).

In glioma cells, the antiproliferative effects of Ad.*mda*-7 were enhanced by IR in a greater than additive manner (37). The radiosensitizing effects were not observed in non-transformed primary human fetal astrocytes. Growth inhibition of glioma cells *in vitro* correlated with an increased number of cells in the G_1/G_0 and G_2/M phases of the cell cycle, confirming cell cycle independence of the radiosensitization effect (61). Irradiation of cells, but not Ad.*mda*-7 infection itself, dramatically enhanced JNK 1/2 activity. Accordingly, inhibition of JNK 1/2 with its specific inhibitor SP600125 abolished radiosensitization properties of MDA-7/IL-24. Both Ad.*mda*-7 and recombinant GST-MDA-7/IL-24 protein demonstrated radiosensitizing properties in malignant glioma cell lines, and this effect correlated with increased expression of specific members of the *GADD* gene family, particularly, *GADD153* (37). Similarly, antiproliferative and cytotoxic properties of recombinant GST-MDA-7/IL-24 protein were enhanced by radiation in primary (non-established) human glioma cells (38). *In vivo*, Ad.*mda*-7 enhanced the survival of Fischer 344 rats implanted intracranially with rat malignant glioma cells. Radiation did not alter survival in control-infected

animals, whereas it prolonged survival in animals infected with Ad.*mda*-7 (26).

GST-MDA-7/IL-24 protein also demonstrated radiosensitizing effect *in vitro* in breast cancer cells MDA-MB-231 (53). Moreover, ectopic *mda*-7/IL-24 expression (or treatment with purified recombinant GST-MDA-7/IL-24 protein) in combination with IR was able to overcome both radioresistance and *de novo* resistance to *mda*-7/IL-24-induced apoptosis in prostate cancer cells overexpressing Bcl-2/Bcl-x_L (40). JNK activation was documented to be a central event in the radiosensitization of cancer cells and blocking of JNK activation ameliorated this radiation enhancement effect (40). Considering the fact that elevated expression of Bcl-x_L and Bcl-2 are frequent events in prostate cancer development and progression, the results of this study support the use of IR in combination with *mda*-7/IL-24 to augment the therapeutic benefit of this gene in prostate cancer, particularly in the context of tumors displaying resistance to radiation therapy owing to Bcl-2 family members overexpression.

In NSCLC, Ad.*mda*-7 infection enhanced radiosensitivity independently of the *p53* status of the cells and independently of any ability to up-regulate the expression of Fas or BAX. Phosphorylated c-Jun expression was demonstrated in NSCLC cell lines treated with both IR and Ad.*mda*-7, but not in normal human lung fibroblasts, CCD-16 (62). Nishikawa *et al* (23) demonstrated that not only did Ad.*mda*-7 infection in combination with radiation enhance apoptosis in tumors, but also that secreted MDA-7/IL-24 protein sensitized HUVEC cells to IR thus inhibiting angiogenesis. Further studies revealed that Ad.*mda*-7 sensitizes NSCLC cells to IR by suppressing the activity of non-homologous end-joining pathway of double strand break repair (68). A recent study by Chada *et al* (58) described a synergistic effect of the combination of Ad.*mda*-7 and IR on breast cancer cells *in vitro* and *in vivo*, however, the mechanism of this effect was not investigated.

Emdad *et al* (65) demonstrated that *mda*-7/IL-24-induced apoptosis in ovarian cancer cells could be significantly enhanced by combination with ionizing radiation. Additionally, *mda*-7/IL-24 gene delivery under the control of a minimal promoter region of progression elevated gene-3 (*PEG*-3), which functions selectively in diverse cancer cells with minimal activity in normal cells (69-74), displayed a selective radiosensitizing effect in ovarian cancer cells (65).

In summary, studies in multiple human tumor model systems, including malignant glioma, and carcinomas of the lung, breast, prostate and ovary (26,37,38,40,58,61,62,65), suggest that the therapeutic index of *mda*-7/IL-24 can be enhanced by its use in combination with IR. The ability of secreted *mda*-7/IL-24 to inhibit tumor angiogenesis, which is promoted by IR, provides another reason for using this combinatorial approach for cancer therapy. Of additional importance, the ability of the combination of *mda*-7/IL-24 and IR to reverse resistance in specific cancers, such as prostate carcinoma cells overexpressing Bcl-2 or Bcl-x_L, to either agent used alone, suggests expanded applications of this novel cytokine in the context of potentially resistant tumor cells. Further studies are necessary to confirm improved antitumor activity in patients by combining *mda*-7/IL-24 with IR.

4. Ectopic expression of *mda*-7/IL-24 inhibits invasion and migration of cancer cells

Abnormalities in smooth muscle cell (SMC) proliferation and differentiation underlie the pathogenesis of proliferative vascular diseases. Due to *mda*-7/IL-24's involvement in apoptosis and differentiation processes in diverse cancers, this gene was used to modulate SMC growth and migration (75). Overexpression of *mda*-7/IL-24 suppressed growth of PAC1 rat cells (exhibiting multiple chromosomal aberrations) and induced apoptosis in this cell line in a dose-dependent manner; this treatment had no effect on normal primary human coronary artery cells or rat aortic SMC despite the high levels of MDA-7/IL-24 expression in all SMC. Ad.*mda*-7 also inhibited serum-stimulated PAC1 cell migration. In contrast, recombinant MDA-7/IL-24 protein did not elicit death or STAT3 activation in PAC1 cells that lacked IL-20/IL-22 receptors suggesting that the effects of Ad.*mda*-7 were mediated through intracellular pathways.

When applied to human non-small cell lung carcinoma cells (NSCLC), Ad.*mda*-7 inhibited migration and invasion of H1299 and A549 cells (76). Mechanistic studies revealed down-regulation of PI3K/PKB, FAK and MMP-2 and -9 following Ad.*mda*-7 infection. Furthermore, tumor cells treated with Ad.*mda*-7 *ex vivo* or with DOTAP:Chol-*mda*-7/IL-24 complex *in vivo* formed significantly less tumors in an experimental lung metastasis model. Both migration and invasion processes played pivotal roles in cancer metastasis. Thus, MDA-7/IL-24-based drugs may provide a novel therapeutic strategy that can inhibit tumor growth directly by inducing apoptosis, and may also prevent tumor invasion, ultimately reducing metastasis.

Sauane *et al* (45) compared the effect of secreted and non-secreted forms of MDA-7/IL-24 on C8161 tumor cell invasiveness. The C8161 cell line is characterized by very high invasive capacity *in vitro* and by tumorigenic and metastatic properties *in vivo* in nude mice (77). Infection of C8161 cells with Ad.SP-*mda*-7 (an adenovirus producing a version of *mda*-7/IL-24 that is not secreted because of the absence of the secretory signal peptide at amino acids 1-49) or with Ad.*mda*-7 inhibited to a similar extent the ability of these cells to invade through Matrigel-coated membrane inserts as compared with Ad.*vec* infected cells. In these experimental settings, no effect was observed on C8161 cell growth in monolayer, thereby confirming that invasiveness was not inhibited because of loss of cell viability (45).

Moreover, MDA-7/IL-24 secretion by normal cells inhibited invasion of cancer cells expressing the complete set of IL-20/IL-22 receptors, such as DU-145 or BxPC-3, but not A549 cells that lack a full complement of IL-20/IL-22 receptors (35). In these experiments, P69 immortalized normal human prostate epithelial cells were infected with Ad.*mda*-7 and cocultivated with different cancer cells for 48 h and then evaluated for invasion. Under these conditions, no decrease in cell growth or viability was evident, and no apoptosis was induced in these cancer cells (35). These results indicate 'antitumor (invasion inhibition) bystander effect' of secreted MDA-7/IL-24 protein can be expected to enhance the therapeutic properties of this cytokine and promote biologically relevant effects at a distance from its initial cellular site of synthesis and secretion.

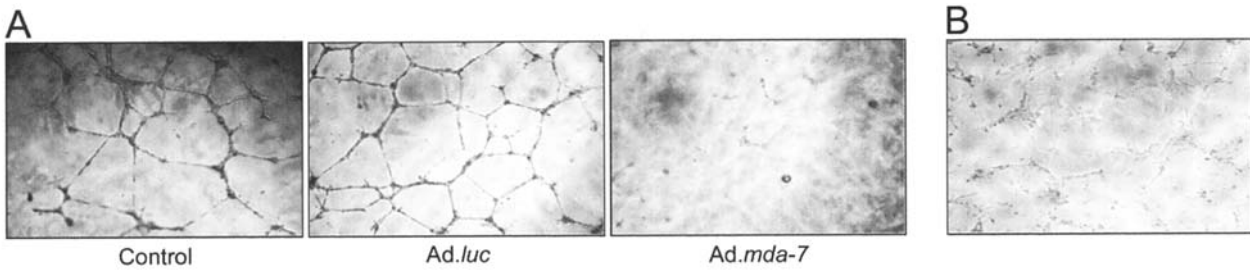

Figure 3. Demonstration of *mda*-7/IL-24-mediated antiangiogenic activity by tumor-endothelial cell mixing experiment *in vitro*. A, Human lung tumor cells (H1299) treated with PBS, Ad.*luc* or Ad.*mda*-7 were plated in 96-well plates and overlayed with Matrigel. HUVEC seeded on top of the Matrigel were analyzed for ECD or tube-formation. Significant inhibition of ECD was observed in wells that contained Ad.*mda*-7 treated tumor cells compared to PBS or Ad.*luc* treated tumor cells. B, Photomicrograph showing H1299 cells at the bottom of the Matrigel coated wells.

5. Antiangiogenic activity of *mda*-7/IL-24

Angiogenesis, a complex process that involves many cell types and a finely orchestrated series of events that include endothelial cell proliferation, new capillary formation, attraction of pericytes and macrophages, disruption of existing extracellular matrix and deposition of new matrix, is a critical process required by solid tumors to support their growth (78,79). The role of angiogenesis in growth and metastasis of tumors is well established and recent studies support the concept that metastasis from solid tumors is facilitated by angiogenesis of the primary tumor (80). In these contexts, inhibition of tumor angiogenesis should prove to be an effective means of inhibiting cancer growth and spread.

A number of angiogenesis inhibitors have been identified and found to block tumor growth by inhibiting tumor angiogenesis in preclinical studies (81-91). Angiogenesis may be regulated on multiple levels: some inhibitors may function to impede the formation of new blood vessels (antiangiogenic), whereas others may disrupt or modify existing vessels (antivascular). Although several antiangiogenic molecules have been developed and tested only a few have demonstrated a therapeutic effect in clinical trials (81,82). Based on the potential relevance of this approach and its limited current success, there has been a need to identify and characterize new and novel agents that target the tumor and its vasculature. Below, we summarize preclinical findings showing the antiangiogenic activity of *mda*-7/IL-24.

Initial studies testing the antitumor activity of *mda*-7/IL-24 using an adenoviral vector (Ad.*mda*-7) demonstrated that Ad.*mda*-7 was cytotoxic to a panel of human lung tumor cells, but not to endothelial cells *in vitro* (24). However, *in vivo* molecular analysis of the growth inhibitory effects showed Ad.*mda*-7-treated tumors had reduced tumor vascularization compared to control vector-treated tumors (24). These results suggested that the reduced tumor vascularization in Ad.*mda*-7-treated tumors could be either due to direct tumor killing or antiangiogenic activity. To test whether *mda*-7-mediated an antiangiogenic activity, *in vitro* and *in vivo* experiments were conducted. In *in vitro* studies, Ad.*mda*-7-treated human H1299 lung tumor cells that served as an ectopic source for MDA-7/IL-24 protein were mixed with human umbilical vein endothelial cells (HUVEC) and plated onto Matrigel coated 96-well plates and observed for endothelial cell differentiation (ECD), an assay that is routinely used to test for antiangiogenic activity. Tumor cells that were treated with PBS or infected with Ad.*luc* (vector control) and mixed with HUVEC served as controls. A marked inhibition of ECD was observed in wells containing Ad.*mda*-7-treated tumor cells (Fig. 3). No inhibition of ECD was observed in wells that contained PBS or Ad.*luc*-treated tumor cells. These results showed that tumor cells exogenously expressing MDA-7/IL-24 protein when in close proximity to endothelial cells could inhibit ECD. These observations confirm that *mda*-7/IL-24 has antiangiogenic activity.

Although results from *in vitro* mixing experiments supported *in vivo* findings for *mda*-7/IL-24-mediated antiangiogenic activity, the exact mechanism was not known. Furthermore, previous studies had shown that tumor angiogenesis could be inhibited directly by suppressing tumor blood vessel formation and indirectly by suppressing the production of tumor-derived growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and interleukin-8 (IL-8) (78-81). Thus, it was possible that *mda*-7/IL-24 exerted its antiangiogenic activity by a direct or an indirect mechanism or by a combination of both processes. Confirmation of direct and indirect inhibitory effects of *mda*-7/IL-24 on tumor angiogenesis is discussed below.

Direct inhibitory effect. To test whether *mda*-7/IL-24 directly affected the tumor vasculature, experiments were performed using affinity purified human MDA-7/IL-24 protein (7). *In vitro*, MDA-7/IL-24 protein selectively inhibited ECD with no effect on cell proliferation (92). The inhibitory effect on ECD was dose-dependent with complete inhibition occurring at concentrations above 10 ng/ml. Note, at the concentrations of MDA-7/IL-24 protein used no significant cytotoxicity against receptor-negative lung tumor cells was observed indicating selective activity against receptor-positive endothelial cells. That the inhibitory effect was specific to MDA-7/IL-24 protein was demonstrated by immunodepletion studies (92). Additionally, the inhibitory effect of MDA-7/IL-24 protein on ECD was 10-50 times more potent than endostatin, interferon-γ and interferon-inducible protein-10 when tested at equimolar concentrations *in vitro* (92). Additional studies showed that MDA-7/IL-24 protein effectively inhibited in a dose-dependent manner VEGF and FGF-induced endothelial cell migration (92). These results demonstrate direct antiangiogenic activities for the MDA-7/IL-24 protein.

Molecular analysis of MDA-7/IL-24 protein-mediated antiangiogenic activity showed a requirement for IL-22R1, one of the two receptors for MDA-7/IL-24 (32,33,92). The

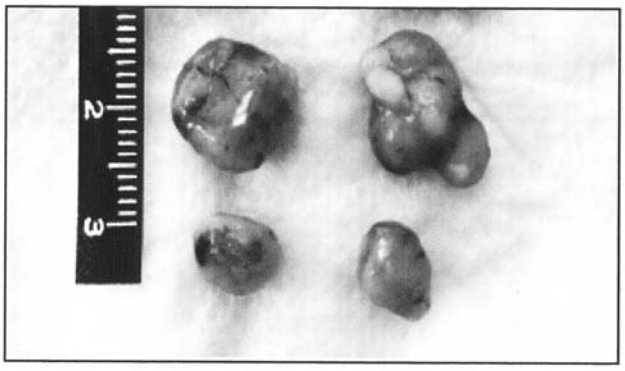

Figure 4. Antiangiogenic activity of *mda*-7/IL-24 *in vivo*. Human A549 lung tumor cells were mixed with parental 293 cells (A549 + 293) or mixed with 293-*mda*-7 cells (A549 + 293-*mda*-7) and implanted subcutaneously into the lower right flank of nude mice. Tumor growth was measured at regular intervals and experiments terminated after 3 weeks. A significant inhibition of tumor growth was observed in mice implanted with a mixture of A549 + 293-*mda*-7 cells (bottom) compared to mice implanted with a mixture of A549 + 293 (top).

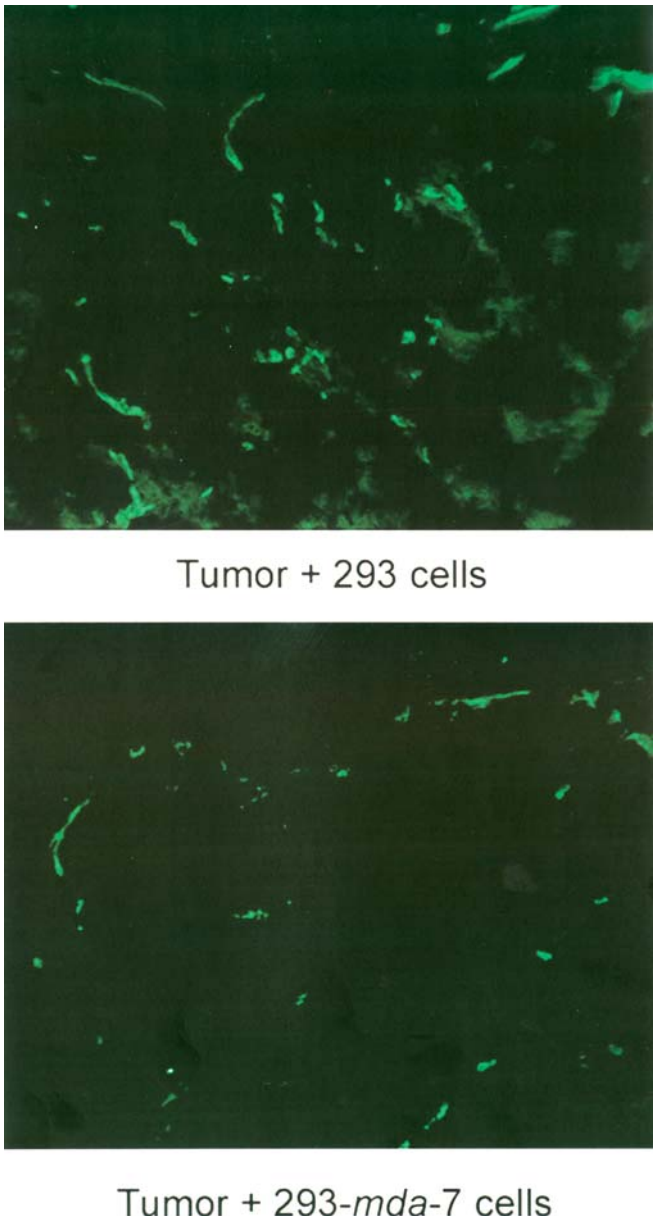

Figure 5. Systemic inhibition of tumor vascularization by *mda*-7/IL-24. Subcutaneous A549 lung tumor xenografts were established in nude mice in the lower right flank. When the tumors were measurable, Matrigel encapsulated 293 or 293-*mda*-7 cells were implanted in the upper right flank. At 3-4 weeks after initiation of the experiment animals were euthanized, tumors harvested and tissue sections prepared, and stained for CD31 using FITC-conjugated antimouse CD31 antibody. A significant decrease in CD31 positive blood vessels was observed in tumors that were obtained from mice implanted with 293-*mda*-7 cells compared to tumors that were obtained from mice implanted with 293 cells.

involvement of IL-22R for MDA-7/IL-24 protein-mediated antiangiogenic activity was demonstrated by activation of STAT-3 in HUVEC as a measure of receptor-ligand interaction and by receptor-blocking studies (92). Although involvement of IL-22R in MDA-7/IL-24 protein-mediated antiangiogenic activity has been demonstrated, the role of the IL-20 receptor in *mda*-7/IL-24-mediated antiangiogenic activity has yet to be determined. Additionally, the downstream signaling mechanisms regulated by *mda*-7/IL-24 in endothelial cells has not been studied. One possibility is the activation of STAT-1, a molecule that is associated with antiangiogenic phenotype (93). Another possibility is *mda*-7/IL-24-mediated inhibition of the PI3K/AKT pathway that is associated with a proangiogenic phenotype. Recent studies have demonstrated PI3K/AKT inhibition in Ad.*mda*-7-treated lung and breast cancer cells (63,94).

Evidence for direct effects of MDA-7/IL-24-mediated antiangiogenic activity *in vivo* has been obtained. Subcutaneous implantation of MDA-7/IL-24 producing 293 cells (293-*mda*-7) mixed with receptor negative A549 lung tumor cells (1:1 ratio) in nude mice demonstrated suppression of tumor growth compared to tumor growth in mice implanted with a mixture of parental 293 cells and tumor cells [Fig. 4; (92)]. Tumor growth inhibition was accompanied by apoptosis of tumor endothelial cells and tumor cells, reduced hemoglobin content, and less CD31 positive endothelial cells (92). These results demonstrate the direct antiangiogenic activity of MDA-7/IL-24 protein.

To test whether MDA-7/IL-24 protein can inhibit tumor growth at a distant site systemically, A549 lung tumor xenografts were established subcutaneously in the lower right flank of nude mice. Subsequently when the tumors had grown to a size of 50-100 mm^3, Matrigel containing parental 293 cells or Matrigel containing 293-*mda*-7 cells were implanted subcutaneously into the upper right flank of tumor-bearing animals and the effects of *mda*-7/IL-24 on tumor growth were measured. A significant growth inhibition with 40-50% reduction in tumor size was observed in mice that were implanted with Matrigel containing 293-*mda*-7 cells compared to mice that were implanted with Matrigel containing 293 cells (92) (Fig. 5). That the observed tumor growth inhibition was due to systemic inhibitory effects of MDA-7/IL-24 protein on tumor angiogenesis was demonstrated by detecting the protein in the blood and a reduction in CD31 positive blood vessels (92). Furthermore, no gross pathological changes were observed in the animals implanted with 293-*mda*-7 cells suggesting that the protein had no toxic side effects. These results demonstrated MDA-7/IL-24 protein systemically inhibits tumor growth by inhibiting tumor angiogenesis. Although demonstration of the antiangiogenic activity of *mda*-7/IL-24 is exciting, it should be noted that

these studies were conducted in an immunodeficient nude mouse model. It is of interest to test the systemic effects of *mda*-7/IL-24 on tumor angiogenesis in an immunocompetent mouse model as well as to evaluate for toxicity.

Intratumoral or systemic administration of *mda*-7-nanoparticles into immunocompetent C3H mice bearing syngeneic UV2237m subcutaneous tumors or experimental lung metastases resulted in tumor growth inhibition (20,76). Associated with growth inhibition was the demonstration of reduced vascularization demonstrating *mda*-7/IL-24-mediated antiangiogenic activity. Additionally, no measurable toxicity was observed. The results from this study showed that the antitumor activity of *mda*-7/IL-24 in immunocompetent tumor-bearing mice is partly mediated by inhibiting angiogenesis with no toxicity, a phenomenon akin to that seen in immunodeficient tumor-bearing mice.

Indirect inhibitory effect. Although direct antiangiogenic activity for MDA-7/IL-24 protein was demonstrated (92), MDA-7/IL-24 like many other antiangiogenic agents can also indirectly exert its antiangiogenic activity by inhibiting tumor-derived growth factor (IL-8, bFGF, VEGF) expression (95-97). Thus, inhibition of these growth factors will result in the suppression of tumor growth due to its inability to support neovascularization. Supporting this possibility is the recent report by Nishikawa *et al* (23) who showed inhibition of VEGF, bFGF, and IL-8 in Ad.*mda*-7-treated human lung tumor xenografts. In the same study, combining radiation therapy with Ad.*mda*-7 documented enhanced radiosensitization of lung tumor xenografts that was associated with a significant inhibition of VEGF, bFGF, IL-8 and tumor neovascularization. In fact, combination therapy showed greater inhibition of these proangiogenic factors compared to radiation or Ad.*mda*-7 treatment alone. Thus, a role for indirect antiangiogenic activity for *mda*-7/IL-24 was established. However, the molecular mechanism by which *mda*-7/IL-24 exerted its inhibitory activity on these growth factors was neither examined nor reported.

Recent studies demonstrate that *mda*-7/IL-24 inhibits VEGF expression at the transcriptional level resulting in reduced VEGF protein expression [Fig. 6; (98)]. Molecular analysis revealed that Src kinase regulated VEGF transcription and protein expression and that *mda*-7/IL-24 directly inhibited Src kinase activity, thereby resulting in reduced VEGF transcription and expression. Additionally, tissue culture supernatant collected from *mda*-7/IL-24-treated tumor cells that contained reduced VEGF, as determined by ELISA, when added to actively growing endothelial cells showed reduced VEGFR2 signaling and Akt activation leading to inhibition of cell proliferation and induction of apoptosis (98). In contrast, addition of culture supernatants from tumor cells that were treated with PBS or Ad.*luc* (vector control) and contained high levels of VEGF resulted in increased endothelial cell proliferation that was associated with increased VEGFR2 and Akt activation. Thus, *mda*-7/IL-24-mediated inhibition of VEGF expression in tumor cells results in the inability of the tumor to support endothelial cell survival. These results showed that *mda*-7/IL-24 could inhibit angiogenesis indirectly by inhibiting tumor-derived growth factor expression, such as VEGF. Additional growth factors that were inhibited by *mda*-7/IL-24 include IL-8, FGF, and TGF-ß (Inoue *et al*, unpublished data). In conclusion, *mda*-7/IL-24 exerts its antiangiogenic activity both by direct and indirect mechanisms that are mediated by the extracellular and intracellular protein, respectively.

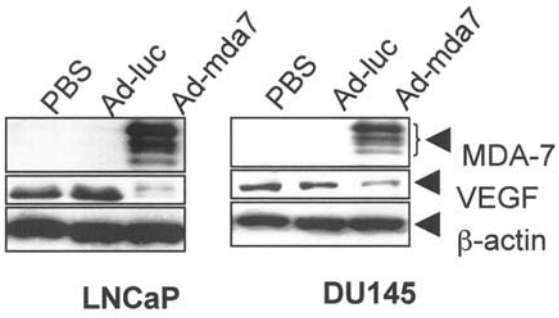

Figure 6. Inhibition of VEGF by *mda*-7/IL-24. Human prostate tumor cells (LNCaP and DU-145) seeded in 6-well tissue culture plates were treated with PBS, Ad.*luc* or Ad.*mda*-7. At 72-h after treatment, cells were harvested, cell lysate prepared and subjected to Western blotting for VEGF protein expression. Inhibition of VEGF that correlated with MDA-7/IL-24 protein expression was observed in Ad.*mda*-7-treated tumor cells and not in PBS- or Ad.*luc*-treated cells. VEGF was inhibited in both LNCaP and DU-145 tumor cells. ß-actin was used as a loading control.

6. Immune properties of *mda*-7/IL-24

Several features of *mda*-7/IL-24 characterize this gene as a cytokine and a member of the IL-10 family of cytokines. These features include chromosomal location on chromosome 1q31-32 in a region containing a cluster of IL-10 family member genes (3,5), translational regulation (42,44,99-101), a secretory motif, and an IL-10 signature motif. Several experimental studies document that MDA-7/IL-24 protein can be secreted and that purified MDA-7/IL-24 protein can bind to IL-20R1/IL-20R2 and IL-20R1/IL-22 receptor complexes and activate STAT-signaling pathways (7,12,34,35). Physiological expression of the *mda*-7/IL-24 gene occurs in a highly restricted manner in human cells, and associates with cells having immunological functions (5,7). Several studies show expression of *mda*-7/IL-24 both on an mRNA and protein level in melanocytes and a progressive loss of expression in melanoma (2,5,10-13). Loss of *mda*-7/IL-24 expression in progressing melanoma might contribute to the ineffective immune response to melanoma (102).

Earlier studies based on multiple tissue Northern blots containing poly(A) RNAs from normal human tissue demonstrated *mda*-7/IL-24 tissue specific expression in thymus, spleen and peripheral blood mononuclear cells (PBMCs) compared to several other tissues (5). Two broad classes of cells were shown to endogenously express *mda*-7/IL-24, including melanocytes and subpopulations of PBMCs. These included normal unstimulated or LPS stimulated monocytes, anti-CD3 stimulated T-cells, and normal human melanocytes (5,7,9).

A potential role for *mda*-7/IL-24 as a cytokine and its involvement in the immune system has been highlighted in two studies (7,9). *Mda*-7/IL-24 expression could be induced in PBMCs upon treatment with LPS or PHA (7). In addition, *mda*-7/IL-24 mRNA could be detected by real-time PCR in monocytes and its expression was up-regulated in monocytes

by treatment with LPS or in T-cells, especially in $CD4^+$ naïve and memory cells, following activation by anti-CD3 mAb (9). No expression has been shown in NK or B-cells before or after stimulation. Further studies in T-cells demonstrated that at earlier time points (6 h), *mda*-7/IL-24 expression was down-regulated in cells undergoing T1 differentiation (mediating cellular immunity) and slightly up-regulated in cells undergoing T2 differentiation (regulating humoral immunity). At 66 h, the expression increased in cells committed to T1 differentiation (9). However, the expression pattern in mouse T cells is different from that in humans. The mouse counterpart of *mda*-7, FISP, was shown to have exclusive expression in Th2 lymphocytes (103).

Treatment of PBMCs with MDA-7/IL-24 protein, purified from conditioned media of a 293-cell line stably expressing *mda*-7/IL-24, resulted in the induction of IL-6, IFN-γ, TNF-α, IL-1ß, IL-12, and GM-CSF (granulocyte macrophage-colony stimulating factor) (7). These inductions were blocked, either completely or partially, by simultaneous administration of IL-10, which might occur due to sharing of receptor subunits by IL-10 family and due to a 10-fold higher affinity of IL-10 for its receptors (9). However, treatment with MDA-7/IL-24 did not modulate the proliferative functions of PBMCs (7). It was hypothesized that the secondary cytokines induced by MDA-7/IL-24 might activate antigen presenting cells to present tumor antigen, thereby triggering an antitumor immune response (7). In PBMC cultures, the cytokine activity of MDA-7/IL-24 was antagonized by the prototypic immunosuppressive cytokine IL-10. These results suggest that even though MDA-7/IL-24 and IL-10 are related family members and share 19% amino acid homology, they bind to different receptors and appear to have opposing roles in regulating immunity against incipient tumors. In melanoma, pathologic progression correlates with increased IL-10 levels (104), therefore IL-10 can function as an autocrine growth factor for melanoma. Expression of IL-10 in melanoma and other tumors may facilitate loss of immune surveillance by blocking tumor antigen presentation and thereby inhibiting cell-mediated immunity (9). In contrast, MDA-7/IL-24 induces cell cycle arrest and death in melanoma cells, therefore *mda*-7/IL-24-based drugs may be especially efficient for melanoma treatment because they restore a protein involved in the genesis and progression of this disease.

Garn *et al* (105) demonstrated expression of *mda*-7/IL-24 in response to stimulation by various members of the IL-10 family of cytokines in mouse and human macrophages. LPS or IL-4 (but not TNF-α) treatment of rat alveolar macrophages resulted in *mda*-7/IL-24 mRNA and protein expression. Induction of *mda*-7/IL-24 mRNA correlated with expression of IL-1, IL-6, and TNF-α in cultured human monocytes infected with influenza virus confirming a proinflammatory role for this molecule.

More recent studies of *mda*-7/IL-24 induction in human PBMCs stimulated by PHA and LPS or by allogeneic major histocompatibility complex confirmed data presented by Caudell *et al* (7), Wolk *et al* (9), and Garn *et al* (105) and demonstrated that the expression of *mda*-7/IL-24 in human PBMCs results from cytokine stimulation and is regulated at the post-transcriptional level through stabilization of *mda*-7/IL-24 mRNA (106). Individual cytokines including IL-2, IL-7, IL-15, TNF-α, GM-CSF and IL-1ß stimulate the expression of *mda*-7/IL-24 mRNA and protein, whereas interferons and Th2 type cytokines fail to induce substantial *mda*-7/IL-24. Since cytokines stimulating *mda*-7/IL-24 production were secreted from cells involved in an inflammatory response, the authors hypothesized that *mda*-7/IL-24 might play an important role in the inflammatory response in the skin occurring as a result of a danger signal initiated by viral or bacterial infection or tumor cell growth. Even though MDA-7/IL-24 protein induced inflammatory cytokines and activates inflammatory and apoptotic signaling proteins, it did not activate NF-κB alone, but significantly enhanced TNF-α-induced NF-κB activation and NF-κB-regulated expression of cyclin D1 and COX-2 (107). Potentiation of TNF-α-induced NF-κB activation by MDA-7/IL-24 may occur through MDA-7/IL-24-induced PKR activation, since it is known that PKR activates NF-κB by phosphorylation of IκBα (108) or by interacting with IKK (109,110). The effects described by Aggarwal *et al* (107) appear to be receptor-independent.

In addition to direct tumor cell killing of transformed human and rodent cells (14,17,18), *mda*-7/IL-24 gene transfer can also elicit immunotherapeutic activity against syngeneic murine melanoma tumors (111). UV2237m murine fibrosarcoma cells infected with Ad.*mda*-7 were not able to grow after injection into syngeneic immunocompetent C3H mice, in contrast with immunocompromised nude mice, in which all mice developed tumors after the injection. The authors of this study hypothesized that Ad.*mda*-7 treatment of tumors would result in local MDA-7/IL-24 protein secretion, which would induce or promote systemic antitumor immunity. As predicted, when challenged with parental tumor cells, these tumor-free C3H mice that previously received Ad.*mda*-7/IL-24 treatment experienced no tumor growth. Splenocytes prepared from vaccinated C3H mice demonstrated higher proliferative activity and produced elevated levels of TH1 cytokines, such as TNF-α, GM-CSF, IL-6 and IFN-γ. These findings corroborate the *in vitro* data of Caudell *et al* (7), which showed induction of TH1 cytokines in human PBMCs following treatment with purified human MDA-7/IL-24 protein. Production of IFN-γ, TNF-α and IL-6 was also reported in the serum of patients treated intratumorally with Ad.*mda*-7/IL-24 (27). An *in vitro* subset analysis of splenocytes from vaccinated mice demonstrated a significant increase in the $CD3^+CD8^+$ but not the $CD3^+CD4^+$ cell population. These data are also in agreement with the results from a recently finished clinical trial where patients that received an intratumoral injection of Ad.*mda*-7 (INGN 241) experienced a marked increase in $CD3^+CD8^+$ T-cells after treatment (27). The exact mechanism of *mda*-7/IL-24-induced systemic immunity is still unknown. One possibility is that antigen-presenting cells take up for presentation T-cell immunogenic apoptotic bodies resulting from *mda*-7/IL-24-mediated apoptosis. Another possibility considers activation of immune accessory and effector cells present within the tumor microenvironment and at distant sites by secreted MDA-7/IL-24 protein (111).

7. 'Bystander antitumor' activity of *mda*-7/IL-24

Multiple experiments performed *in vitro* and *in vivo* confirm the remarkable ability of *mda*-7/IL-24 to induce apoptosis in

most cancers. However, pancreatic cancer cells display an inherent resistance to the adenovirus-mediated *mda*-7 infection (44). Pancreatic cancer is a complex disease where multiple subsets of genes undergo genetic changes during tumor development and progression. One of the most frequent and earliest genetic alterations in pancreatic cancer (85-95%) involves activation of the K-*ras* oncogene (112). The lack of activity of Ad.*mda*-7 is not a consequence of failure to express *mda*-7/IL-24 mRNA. Rather, it involves a translational suppression in conversion of this mRNA into protein (42,44,99). When expression of mut K-*ras* is down-regulated by means of antisense oligonucleotides, plasmid or adenovirus expressing antisense K-*ras*, pancreatic cancer cells with mut K-*ras* become sensitive to Ad.*mda*-7-induced apoptosis (44). This observation itself provides a basis for developing rational targeted therapies for pancreatic cancer. Furthermore, these original studies using antisense phoshorothioate oligonucleotides or DNA plasmid transfection to down-regulate K-*ras* lead to another important and provocative observation of 'bystander antitumor' activity *in vitro* and *in vivo*. Tumor formation was suppressed when Ad.*mda*-7-infected cells (estimated to be ~100% of the cells) were combined with transfection with an antisense K-*ras* plasmid (likely transducing ~3-5% of the cells). Nevertheless, the growth of the whole population of tumor cells was efficiently suppressed, a phenomenon also observed in the *in vitro* setting. This supports a 'bystander antitumor' effect in which tumor cells receiving both *mda*-7/IL-24 and antisense K-*ras*-induced changes, likely resulting from the secretion of MDA-7/IL-24 protein, that led to cell death in tumor cells with none or only one of these two genetic manipulations. Additional early evidence of 'bystander antitumor' effect derives from experiments where primary rodent hepatocytes were infected with Ad.*mda*-7 resulting in the production and secretion of bioactive MDA-7/IL-24, which when applied to DU-145 cells suppressed their growth *in vitro* (Su *et al*, unpublished data). Understanding the mechanism underlying this 'bystander antitumor' effect could provide new therapeutic insights as well as help in defining ways of improving the anticancer properties of *mda*-7/IL-24 (14).

Evidence for 'bystander antitumor' activity was recently provided *in vivo* in human tumor xenograft nude mouse animal studies (73). A conditionally replication competent adenovirus (CRCA) was produced in which replication (E1A function) was controlled by the cancer-specific promoter derived from the *PEG*-3 gene (71,113) to drive replication in tumor cells and *mda*-7/IL-24 expression was controlled by the cytomegalovirus promoter in the E3 region of the adenovirus, Ad.PEG-E1A-*mda*-7 (73). Human breast carcinoma cells were inoculated into the right and left flanks of athymic nude mice producing tumors at both sites. Tumors that formed in the left side of the animal were injected with Ad.PEG-E1A-*mda*-7 resulting in eradication of this tumor (reflective of a primary tumor) as well as elimination of the tumor on the right side of the animal (reflective of a metastatic state). Additionally, injection with a non-replicating Ad.*mda*-7 into the left tumor resulted in tumor regression and a statistically significant decrease, although not a complete eradication, was also evident in the right tumor, confirming 'bystander antitumor' activity of the secreted MDA-7/IL-24. The potent inhibitory effects on distant tumors could be explained by a direct action of the secreted cytokine following interaction with its cognate IL-20/IL-22 receptors that activate signal transduction pathways mediating antitumor activity (34,35). Alternatively, the potent 'bystander antitumor' effect of MDA-7/IL-24 on distant tumor cells may include stimulation of NK cells, since athymic nude mice used in the experiments still have a spleen and a liver and display potent NK cells activity (114).

Three recent studies investigated the mechanism(s) of the 'antitumor bystander' effect exerted by MDA-7/IL-24 (34,35, 115). Transduction of both tumor and normal cells with Ad.*mda*-7 gene results in secretion of glycosylated MDA-7/IL-24 protein (reviewed in refs. 4,14-16,18). While Ad.*mda*-7 and GST-MDA-7 recombinant protein were able to selectively kill cancer cells in a receptor-independent manner involving both a secretory and non-secretory mode of action (45,53), the ability of secreted MDA-7/IL-24 to suppress tumor cell growth, inhibit invasion and radiosensitize cancer cells appears to be receptor-dependent. As a cytokine belonging to the IL-10 family, secreted MDA-7/IL-24 protein can bind to two distinct type II cytokine heterodimeric receptor complexes, type 1 IL-20R (IL-20R1/IL-20R2), and type 2 IL-20R (IL-22R1/IL-20R2) (32,33). Ligand-mediated assembly of both receptor chains rapidly activates STAT3 and, to a lesser extent, STAT1 (30). In melanoma cells, both type 1 and type 2 IL-20R mediate transient STAT3 activation by secreted MDA-7/IL-24 protein. This activation is inhibited by >90% by anti-MDA-7/IL-24 neutralizing antibodies (34). Although MDA-7/IL-24 induces STAT3 activation and cytotoxicity in melanoma cells, these events are uncoupled. Moreover, even though all of the IL-10 family members tested elicited STAT3 activation in melanoma cells, MDA-7/IL-24 was the only cytokine that produced cell death. Using antibodies against type 1 IL20R and type 2 IL20R, Chada *et al* (34) demonstrated, also that MDA-7/IL-24-induced apoptosis in melanoma cells is mediated by type 1 IL-20R receptor, even though engagement of both type of receptors resulted in STAT3 activation. The authors hypothesized that the downstream signaling between MDA-7/IL-24 and other members of the IL-10 family differs or that MDA-7/IL-24 may use a co-receptor not utilized by other family members to activate apoptosis signaling. In a similar study, Zheng *et al* (115) demonstrated that apoptosis induced by exogenous MDA-7/IL-24 protein in breast cancer cells is mediated through IL-20R1/IL-20R2 heterodimeric receptor complex and is independent of STAT3 phosphorylation. Coadministration of the related cytokine IL-10 inhibited MDA-7/IL-24-induced killing and MDA-7/IL-24-induced up-regulation of the tumor suppressor proteins *p53* and $p27^{Kip1}$ (115).

Su *et al* (35) provided evidence of a 'bystander antitumor' effect of *mda*-7/IL-24 resulting from secretion of this cytokine by normal cells. Normal cells were infected with Ad.*mda*-7 and directly co-cultured with cancer cells or co-cultured with cancer cells that were separated by an agar overlay, providing a diffusion model for testing for cytokine efficacy. Cell viability and apoptosis (direct co-cultivation), anchorage independent growth (agar diffusion), radiosensitivity (agar diffusion) and invasion (direct co-cultivation) of cancer cells *in vitro* were evaluated using appropriate assays. Primary and immortalized normal cells (PFHA, FM-516SV and P69)

were infected with Ad.*mda*-7 resulting in the production and secretion of MDA-7/IL-24 protein, which modulated the anchorage-independent growth, invasiveness, survival and radiosensitivity of cancer cells that contained functional IL-20/IL-22 receptors, but not cancer cells that lacked a complete set of receptors. Moreover, the combination of radiation and MDA-7/IL-24 secreted from normal cells evoked a 'bystander antitumor' effect not only in cancer cells inherently radiosensitive or permissive for *mda*-7/IL-24 killing, but also in tumor cells overexpressing the antiapoptotic proteins BCL-2 or BCL-x_L and displaying resistance to either treatment alone (35,40). Furthermore, since this effect can occur even in the presence of small amounts of secreted MDA-7/IL-24 protein (that are barely detectable by Western blotting using polyclonal antibodies to MDA-7/IL-24), secretion of this cytokine from normal cells should have a profound impact on tumor growth and radiosensitization *in vivo* by directly affecting tumor cell survival and by inhibiting angiogenesis. As described above, the recent study by Su *et al* (35) highlights the significance of normal cells as contributors to the potent antitumor activity of MDA-7/IL-24. A novel application of this cytokine to enhance its cancer therapeutic efficacy could exploit normal cells as a repository for producing low levels of MDA-7/IL-24 protein to facilitate both localized and systemic cancer therapies. If successful and non-toxic *in vivo*, constitutively expressing low levels of *mda*-7/IL-24 would allow a maximization of benefit of this novel anticancer gene therapy, potentially providing a means of preventing, in real-time, tumor and metastasis development.

As described previously, another potential mechanism underlying *mda*-7/IL-24-mediated 'bystander antitumor' effect, including inhibition of cancer cell growth in distant tumors in an *in vivo* context, might be activation of the immune system. *Mda*-7/IL-24 expression is restricted to melanocytes and those tissues associated with the immune system, such as spleen, thymus, and PBMCs. Expression of this gene induced in PBMCs with purified recombinant MDA-7/IL-24 protein resulted in the induction of multiple potent immunomodulatory agents such as IL-6, IFN-γ, TNF-α, IL-1ß, IL-12, and granulocyte macrophage-colony stimulating factor (7,27). These secondary cytokines induced by *mda*-7/IL-24 might activate antigen-presenting cells to present tumor antigens, thereby triggering an antitumor immune response. Studies in a phase I clinical trial involving intratumoral injection of Ad.*mda*-7 (INGN 241) suggest that these *in vitro* effects are recapitulated in the context of patients supporting the immune modulating properties of this cytokine (27). Although current studies were performed in athymic nude mice that are immunocompromised, these mice still have a spleen and a liver and display potent natural killer activity (74,114).

Another aspect of the 'bystander antitumor' activity of *mda*-7/IL-24, also elaborated on previously, relates to its potent antiangiogenic activity (24,92). This possibility is exemplified by studies by Nishikawa *et al* (23) using A549 non-small cell lung carcinoma cells that lack a complete set of IL-20/IL-22 receptors. Established tumors in animals were injected with Ad.*mda*-7 and then exposed to radiation. Histological examination of tumors revealed a significant reduction in angiogenic factors (bFGF, VEGF) and microvessel density with enhanced apoptosis in tumors receiving the combination of Ad.*mda*-7 and radiation. Analysis of clonogenic survival of HUVECs, A549 and normal human lung fibroblasts pretreated with conditioned medium from 293 cells stably expressing *mda*-7/IL-24 indicated sensitization of the HUVECs to radiation without affecting A549 or normal human lung fibroblasts. These results confirm that a significant enhancement in killing of A549 tumor cells was observed that was a consequence of secreted MDA-7/IL-24 that was inhibiting HUVECs and tumor angiogenesis. These results suggest increased antitumor activity of Ad.*mda*-7 when combined with radiation in non-small cell lung carcinoma, as has also been shown in malignant gliomas and prostate and breast cancers (26,37,38,40,53,61).

A seminal proof of the 'bystander antitumor' effects of *mda*-7/IL-24 derives from the recent phase I clinical trial (27,28). Administration of Ad.*mda*-7 (INGN 241) intratumorally in patients with advanced carcinomas and melanomas resulted in apoptosis in tumor cells receiving the *mda*-7/IL-24 transgene as well as death in tumor cells not initially receiving the virally-transduced gene. Immunohistochemical staining demonstrated that ~30% of the tumor mass received the *mda*-7/IL-24 gene, however, ~70% of the tumor cells showed detectable apoptosis. This can reflect both a direct effect of secreted MDA-7/IL-24 or an immune-mediated antitumor response, or a combination of both processes.

The ability of a gene therapeutic to effectively manage cancer is dependent upon a number of factors and technical issues. Induction of direct tumor cell killing with the potential to be transmitted in the circulation to elicit an effect not only on a primary tumor, but also metastases, is a profoundly important component required for an effective cancer gene therapeutic. As highlighted in this review, *mda*-7/IL-24 not only induced apoptosis selectively in cancer cells, but it also has immune modulatory, radiation enhancing, antiangiogenic properties and potent 'bystander antitumor' effects, thereby making it an ideal candidate for promoting effective cancer gene therapy (6,7,14,17,24,27,28,35,44,92). Because of these essential qualities as a potential therapeutic gene for cancer, a replication-incompetent adenovirus expressing *mda*-7/IL-24 (Ad.*mda*-7; INGN 241) was evaluated for safety in a phase I clinical trial and based on these results (6,17,27-29), confirming safety with a demonstrable clinical benefit, *mda*-7/IL-24 is currently being evaluated for clinical efficacy in a phase II clinical trial.

8. Translation of *mda*-7/IL-24 into the clinic - A good beginning

Initial clinical testing of the *mda*-7/IL-24 gene as a cancer therapeutic involved a non-replicating adenoviral (Ad) vector deleted in the E1 region (INGN 241) as the delivery vehicle (2,25). The initial trial was designed to prove the principle of mechanistic activity of Ad.*mda*-7 and to demonstrate tolerability. Preclinical models justified single-agent testing of Ad.*mda*-7 (4,6,8,11-13,15-17,22,24,25,37,116). Combination with chemotherapy, radiation therapy, and biological therapy demonstrated no increase in toxic profile of *mda*-7/IL-24-related adverse effects and suggested enhancement in immune-related activity. Preclinical toxicology studies of Ad.*mda*-7 in mice demonstrated adverse effects only at the highest dose

Table I. Patient characteristics and dosing.

Cohort	No. of patients[a]	Age (range)	Previous treatments[b]	Dose (vp)	Adverse events[c]
1	1 (1)	49	S, C, Ca, N, H, G	$2x10^{10}$	2
2	1 (1)	44	S, F, I	$2x10^{11}$	1
3	3 (3)	74 (66-76)	S, RT, T, P, C, V, D, Fl, Cl	$2x10^{12}$	0/1/1
4	3 (3)	45 (38-60)	S, A, C, T, Ta, N, P, RT, E	$2x10^{12}$	0/1/0
5	4 (3)	75 (65-75)	S, Ta, RT, I, D	$2x10^{12}$	0/2/2
6	1 (1)	57	S, A, T, Ta	$2x10^{12}$/divided doses	0
7	7 (5)	86 (76-92)	S, M, V, A, P, T, G, RT, F, I	$2x10^{12}$	2/0/0/2/2
8	8 (5)	64 (62-91)	S, RT, IF, IT, P, T, F	$2x10^{12}$ repeated 2x week for 3 weeks	2/2/2/1/1[d]

[a]No. of patients enrolled per cohort is indicated. The no. of patients completing at least one cycle of treatment is shown in parentheses. [b]Prior treatments: S, surgery; A, adriamycin; C, cyclophosphamide; Ca, capecitabine; Cl, chlorambucil; D, dacarbazine; E, etoposide; F, 5-fluorouracil; Fl, fluradabine; G, gemcitabine; H, herceptin; I, irinotecan; IF, IFN-γ; IT, immunotherapy; M, methotrexate; N, navelbine; P, platinum; RT, radiotherapy; T, taxane; Tz, tamoxifen; V, vincristine. [c]Adverse events possibly or probably related to INGN 241 administration are indicated (≤grade 3). [d]One cohort 8 patient experienced a grade 3 SAE and withdrew from the study.

[$5x10^{12}$ viral particles (vp)/kg; equivalent to a human dose of $3x10^{14}$ vp], which consisted of decreases in body weight, mild liver toxicity, and transient decreases in platelet count (117,118). These results suggest that *mda*-7/IL-24 may be optimally used therapeutically when combined with other treatment modalities (6,14,17-19) and supported initiation of clinical investigation. Thus far, however, clinical investigation has been limited and focused on sorting out mechanistic, pharmacodynamic, and pharmacokinetic issues using monotherapy. A single trial has been completed (28).

Safety. Twenty-two patients with refractory cancer and accessible tissue for surgical biopsy were entered into the first trial with Ad.*mda*-7 (Table I) (28). This was a dose escalation study in which patients received $2x10^{10}$-$2x10^{12}$ vp delivered to the central region of the target tumor. Twenty-two patients completed 1 complete cycle (30 days). Treatment schedules ranged from a minimum of a single injection to two injections per week for 3 weeks. Of the evaluable patients there were 13 females and 9 males. Age ranged from 38 to 92 years, with a median age of 66 years. All patients had been heavily pretreated. Malignant melanoma (21.4%), squamous cell carcinoma of the head and neck (18%), breast carcinoma (14%), and colorectal carcinoma (7%) were the most frequent tumor types treated primarily due to accessibility of injectable tumor lesions. Adverse events were generally mild; transient fever being most common. There was one grade 3 serious adverse event (SAE) involving fatigue in a high dose-treated patient, which was thought possibly to be due to the study drug. The most common mild adverse event specifically related to injection of the study drug was included injection site pain. These effects were seen more consistently at higher doses and delivered volumes of Ad.*mda*-7 and they generally resolved within 48 h after injection. In two patients in the highest dose schedule cohort (twice-weekly injections), marked skin erythema was noted surrounding the injected lesion within 24 h of the injection. This resolved over the next 96 h. A maximum tolerated dose was not attained in this study as the maximum monofacturable concentration was delivered. Overall, Ad.*mda*-7 was well tolerated when administered in single or multidose regimens.

Clinical response. In the first six patient cohorts, injected tissue was excised within 24-96 h after injection. No clinical activity was observed within 24-96 h, although minor changes in morphology in the injected lesions were noted. Three patients receiving the highest single-dose therapy via intratumoral injection exhibited SD (stable disease), with 0, 0, and 23% reduction in tumor size (melanoma, colorectal carcinoma, and SCCHN, respectively). Off-study tumor measurements were taken 5-7 weeks from screening. Time from first injection to death for these patients was 347, 351, and 401 days, respectively.

Eight patients in cohort 8 received injections twice weekly for 3 weeks (in a 28-day cycle for 1 or more cycles); five patients completed at least one cycle of treatment. All patients in this cohort had failed multiple prior therapies (Table II). Two of five patients demonstrated a clinically-significant response to Ad.*mda*-7 injections consisting of at least partial regression of the injected lesion. The most dramatic of these responses was in a 64-year-old female with widely metastatic melanoma (at study entry, she had >10 distinct lesion). Her initial site of treatment was a superclavicular node measuring 2x2 cm at baseline. No appreciable change was noted for the first five injections but by the sixth (and final) injection, a clear decrease in the size of the lesion was apparent and associated erythema was evident. Regression continued over the next 2 weeks until there was no clinical evidence of disease at that site (29) (Fig. 7). Subsequently, a second course of injections was begun on a lesion on the dorsum of her right hand. The baseline measurement was 1.8x2.3 cm and regression was evident by the fifth injection (84% reduction in lesion area). After

Table II. Responses in cohort 8 patients.

Patient	Gender/age	Diagnosis	Date diagnosed	Previous treatments[a]	No. of injections	Response[b]	Time to death[c]
81	M/71	Adenocarcinoma	1998	P, T, G	2[d]	SD	nd
83	F/64	Melanoma	1994	S, RT, IT, IF	24[e]	CR, PR, SD	>600
84	F/62	Melanoma	2000	S, RT, IT, IF	12	PR	309
85	M/64	Penile carcinoma	2002	S, RT, CDDP, T	6	PD	75
86	F/66	NSCLC	2001	RT, P, T	3[d]	SD	180
87	M/62	SCCHN	1992	S, RT, CDDP, T, F, P	6	PD	185
88	M/91	Lip carcinoma	1982	S, RT	6	SD	181

[a]Prior treatments: S, surgery; F, 5-fluorouracil; G, gemcitabine; IF, IFN-γ; IT, immunotherapy; P, platinum; RT, radiotherapy; T, taxane. [b]SD, stable disease; CR, complete response; PR, partial response; PD, progressive disease. [c]Time in days from first injection of INGN 241 until death. [d]Patients 81 and 86 did not complete one full course of treatment (6 injections). [e]Patient received 12 injections on compassionate use protocol.

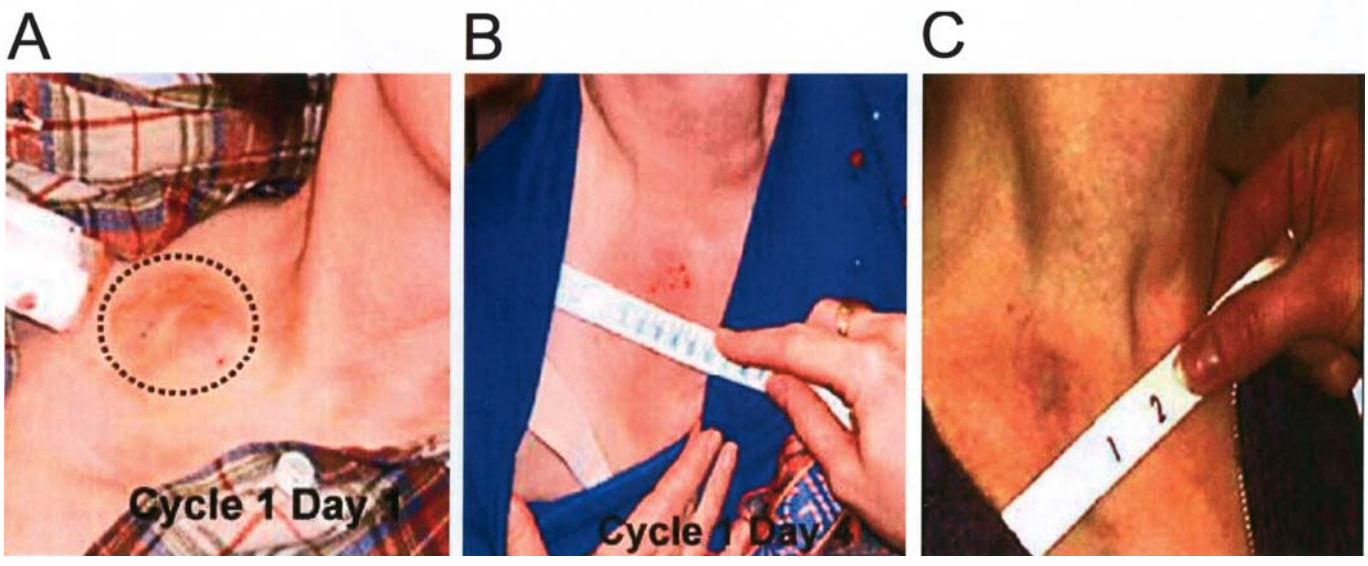

Figure 7. Objective clinical response to INGN 241 (Ad*.mda*-7) in cohort 8 patient with metastatic melanoma (patient 83). Injected lesion was on right clavicle (dashed circle in A). B, By day 4, region is inflamed. C, At the end of cycle 1 (day 30), lesion has completely regressed. This patient is still alive >600 days after initiating treatment.

completing six injections, the residual lesion was excised and on microscopic examination it was found to have marked inflammatory lymphoplasmacytic infiltrates throughout the residual nodule and surrounding tissue. There was also extensive coagulative necrosis in the tumor. A third lesion on the anterior right thigh was next treated in the same patient with two cycles of injections. Regression of this lesion was also observed after the first course of injections (baseline measurements 3.5x3.3 cm; 35% reduction), but a second set of injections produced no further response. Interestingly, several distant uninjected melanoma lesions also became erythematous during the course of injection of the target lesion, although clinical regression was not seen at these distant sites. This patient, a 64-year-old woman, survived 773 days after initiation of treatment with Ad*.mda*-7. An additional melanoma patient exhibited a partial response (33% decrease by RECIST criteria). Five of the eight cohort 8 patients survived more than 2 years, four are still alive at 1643, 1550, 1267, and 1261 days.

We saw a less dramatic response in another patient with squamous cell carcinoma of the penis with multiple skin nodules in the groin and right hip area. Injection of Ad*.mda*-7 into one of the upper right hip lesions (2.5x3.0 cm at baseline) produced significant central necrosis with surrounding erythema by the fifth injection. However, the lesion continued to expand peripherally so that by completion of the first set of six injections, there was an indurated rim of erythematous tissue surrounding the central area and the total measurement of the lesion was 3.0x4.0 cm by the end of the 6th injection. A large central portion of the lesion (approximately two-thirds of the total area) remained blackened and necrotic at the end of cycle 1. Other, new lesions were rapidly appearing in the region, so the patient was removed from the study as PD, and he went on to other therapy. Three additional patients

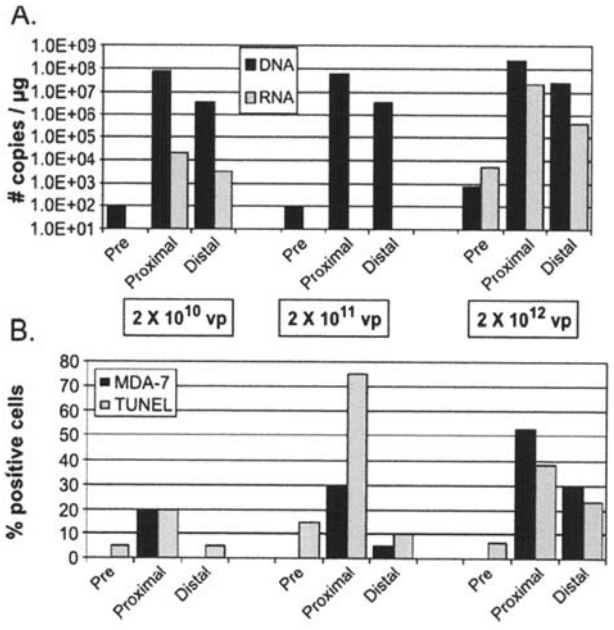

Figure 8. Pharmacodynamics of INGN 241 vector and expression. A, Dose-response of INGN 241 vector DNA and RNA. Tumor sections were obtained from preinjected (pre), proximal and distal sections from cohort 1 to 3 patients and vector-specific signals evaluated by quantitative DNA- and RT-PCR. RNA was not available from cohort 2 tumor. B, Dose-response of INGN 241 transgenic MDA-7/IL-24 expression and TUNEL reactivity. Tumor sections were obtained from preinjected (pre), proximal and distal sections from cohort 1 to 3 patients and vector-specific signals evaluated.

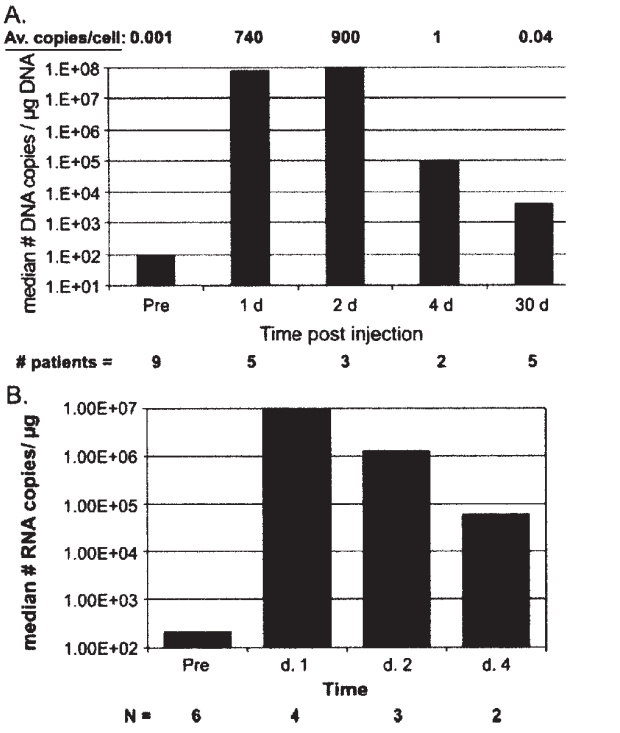

Figure 9. Intratumoral pharmacokinetics of vector DNA and mRNA. A, Decay of INGN 241 vector at injection site. The median number of DNA copies at each time point postinjection is shown; number of patients per sample is indicated below. The number of DNA copies/Ag genomic DNA was converted to illustrate the average number of vector DNA copies per cell - shown above the graph. B, Decay of INGN 241 vector RNA at injection site. The median number of RNA copies/Ag at each time point is shown; number of patients per sample is indicated below.

(with adenocarcinoma, NSCLC, and lip carcinoma) exhibited stable disease after Ad.*mda*-7 injection and one patient with SCCHN exhibited disease progression (20% increase by RECIST). Of the nine lesions treated in cohort 8 patients, four demonstrated objective response (CR or PR by RECIST criteria).

Gene expression. Gene transfer and biologic effects in resected tumors were analyzed 1-4 days after injection. Quantitative studies of vector-specific DNA and RNA at the point of injection (center of lesion) and in serial sections to the periphery of each lesion were performed. To facilitate identification of the injection site, Isosulfan blue dye was admixed with vector prior to administration. Thus, at resection, the injection sites could be identified. All injected lesions showed high levels of vector-specific DNA signals in tumors (Fig. 8). Vector DNA, vector RNA, transgenic MDA-7/IL-24, and TUNEL signals across the dose range of $2x10^{10}$-$2x10^{12}$ vp were compared (Fig. 8). Although the sample size was small, there was an indication of a dose-dependent increase in the number of Ad.*mda*-7 vector DNA copies/μg DNA in the tumor [$7x10^7$ copies/μg in the proximal (central) sections of cohort 1 low dose compared to an average of $2.2x10^8$ copies/μg] in the high dose cohort. A similar indication of dose response for vector RNA was also observed, with $2.0x10^4$ vector RNA copies found in the central section of tumors injected with low dose INGN 241 (Ad.*mda*-7), whereas $2.3x10^7$ copies/μg were observed in high-dose tumors (Fig. 8). Note that the distal regions of tumors uniformly showed lower vector signals than the proximal sections. Parallel sections for transgenic MDA-7/IL-24 protein and apoptosis induction via TUNEL assay were also evaluated. All patients demonstrated undetectable MDA-7/IL-24 staining in the preinjected baseline samples, whereas MDA-7/IL-24 immunostaining was found in 20% of tumor cells from low dose, 30% of cells at intermediate dose, and 53% of cells from tumors injected with high dose of INGN 241. MDA-7/IL-24 staining in distal regions of tumors after injection with low dose vector was not observed, whereas up to 30% positive cells were found in mid- and high-dose tumors. Apoptosis induction showed higher trend with increased dose, except for the cohort 2, patient (colorectal carcinoma), who exhibited unusually high TUNEL reactivity of 75% after injection of Ad.*mda*-7 (Fig. 8B).

Dynamics of vector spread and subsequent gene expression were also evaluated. The highest number of DNA vector copies were found at the area of injection (e.g., the center of injected lesions averaged greater than $1x10^8$ vector DNA copies/μg) and signal decreased significantly ($p<0.02$) with distance from the injection site, vector DNA levels had fallen by almost 90%. Low levels of vector DNA were detected up to 3-cm from the injection site; however, only 3 tumors showed DNA signals greater than $1x10^6$ copies/μg at 1-cm from the injection site. Vector RNA levels were also quantitated and they showed a pattern of distribution similar to that of the vector DNA. Vector RNA was also distributed distally from the injection site and, similar to vector DNA, vector RNA signals were significantly reduced ($p<0.05$) with distance from the injection site. Both vector DNA and RNA signals showed exponential decay with distance from the injection

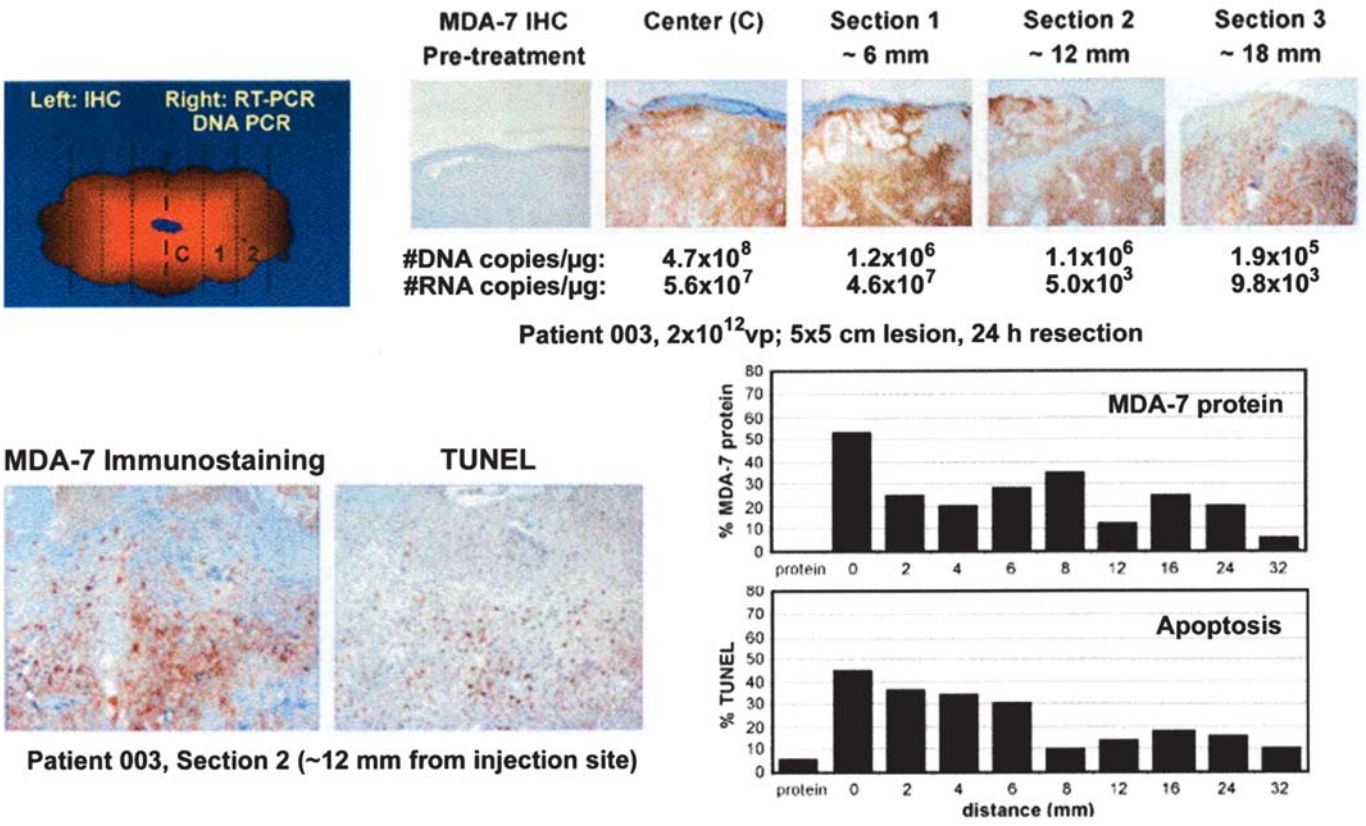

Figure 10. *Mda*-7/IL-24 transgene expression correlates with distribution of vector throughout the tumor. One-half of the tumor was analyzed for MDA-7/IL-24 protein expression and the other half for vector-specific DNA and RNA levels. The number of DNA copies/μg genomic DNA and number of RNA copies/μg total RNA is shown for each tumor section. TUNEL reactivity was 50% in the central section and 30% at the periphery section 3.

site. Regression analyses indicated very strong correlations between vector DNA and RNA signals and distance from injection site (correlation coefficient = 0.82-0.9).

The kinetics of vector DNA and transgenic mRNA were evaluated for patients who received a single dose of INGN 241 (Ad.*mda*-7). Vector DNA reached maximum levels at the point of injection 24-48 h after injection. If one assumes these signals are cell-associated, then median signals approximated 1000 vector DNA copies per cell, a value 6 logs above pre-injection controls (Fig. 9A). Vector-specific mRNA exhibited a distribution and kinetic profile similar to that of vector DNA (Fig. 9B). Median vector-specified RNA signals were more than 4 logs greater in the center of injection lesions compared to uninjected control samples (Figs. 8A and 9B). Vector-specific RNA signals decreased by more than 2 logs by day 4 (Fig. 9B). Samples were not available for day 30 analysis.

Transgenic gene expression. Tumors were excised and serially-sectioned, then evaluated for transgenic MDA-7/IL-24 protein expression and apoptosis. Pretreatment samples were uniformly negative for MDA-7/IL-24 protein expression and most were negative for TUNEL reactivity (average TUNEL signal was <6% in cohorts 1-5). After Ad.*mda*-7 injection, all tumors demonstrated substantial MDA-7/IL-24 immunostaining that ranged from 20 to 90% positive tumor cells in the center of the lesion. Transgenic MDA-7/IL-24 was detected beyond the injection site: five of eight (62%) tumors had detectable MDA-7/IL-24 at 1-cm from the single injection site, and MDA-7/IL-24 expression was detected more than 3-cm from the injection site. Up to 25% of MDA-7/IL-24 stained cells were detected 16-mm from the point of injection. Reproducibly, within each tumor, MDA-7/IL-24 immunostaining was reduced with distance from the injection site. With the exception of patient 2, ≥90% of MDA-7/IL-24 staining cells in all biopsies exhibited malignant histological features. The remaining MDA-7/IL-24-positive cells comprised infiltrating lymphocytes and/or histocytic/reticuloendothelial cells.

Apoptosis staining varied, with up to 80% of tumor cells at the center of the lesion demonstrating TUNEL reactivity. Apoptosis declined with distance from the injection site; five of seven (71%) tumors exhibited TUNEL reactivity beyond 1-cm from the injection site. Both MDA-7/IL-24 and TUNEL staining demonstrated significant ($p<0.02$) linear decay with distance from the injection site. Regions of tumor exhibiting TUNEL staining strongly corresponded to those regions having MDA-7/IL-24 protein expression; those samples with distal MDA-7/IL-24 staining also showed distal TUNEL reactivity. Both protein expression and apoptosis reactivity reached maximum levels by 4-days postinjection but had returned to baseline, preinjection levels by day 30 [(28) and data not shown].

A representative example is shown in Fig. 10, which illustrates the high level of MDA-7/IL-24 immunostaining after Ad.*mda*-7 injection and the decay in protein, DNA, and RNA signals with distance from the injection site. This patient with a 5x5-cm SCCHN lesion was injected with $2x10^{12}$ vp Ad.*mda*-7 and resected at 24 h. No MDA-7/IL-24 signal was

observed in the pretreatment lesion, whereas the injection site (center section) exhibited up to 75% of tumor cells staining for MDA-7/IL-24. Staining intensity decreased with distance, but the distal lesion (18-mm from injection site) still showed regions of strong MDA-7/IL-24 staining (Fig. 10). Parallel sections were evaluated for apoptosis. The central section showed 50% TUNEL reactivity, whereas the distal section showed 30% staining. The levels of vector-specific DNA and RNA showed strong signals at the injection site, which decreased markedly (more than 100-fold) at the periphery (Fig. 10). Overall, vector-specific DNA and RNA, MDA-7/IL-24 protein levels, and TUNEL reactivity demonstrated similar dose response and kinetic and radial concentration gradients, although MDA-7/IL-24 and TUNEL signals persisted longer and showed enhanced distribution.

Conclusions from the phase I clinical trial. Direct injection of an adenovirus containing the *mda*-7/IL-24 cDNA (INGN 241) into a variety of solid tumors was generally well tolerated, with injection site pain and erythema being noted locally in some patients. One patient experienced a grade 3 SAE of fatigue and was discontinued from the study.

Analysis of serial sections of injected lesions demonstrated that *mda*-7/IL-24 DNA and RNA were detectable in 100% of the injected lesions, with the highest concentrations found at the site of injection, as expected. However, MDA-7/IL-24 protein was detectable in the periphery of injected lesions (greater than 3-cm from injection site), beyond the area of DNA spread, suggesting that the MDA-7/IL-24 protein can diffuse from transduced cells, which supports the *in vitro* 'bystander antitumor' studies (18,35,44).

Apoptosis, as measured by TUNEL assay, was significant in the injected lesions and also correlated geographically with MDA-7/IL-24 protein expression. Both MDA-7/IL-24 transgenic protein expression and apoptosis decayed with similar intratumoral dynamics. One hundred percent of injected tumors evaluated in the phase I clinical study demonstrated transgenic MDA-7/IL-24 expression and elevated apoptosis induction compared to untreated control tumors. In contrast, adenoviral-mediated gene transfer of p53 by injection into non-small cell lung cancer tumors resulted in vector detection by DNA PCR in 86% of patients' tumors and increased apoptosis in 46% of biopsy specimens (119). Indeed, as most previous gene transfer studies have not been structured to assess the geographic extent of vector distribution and biological effect, it is difficult to compare INGN 241's effects directly with other studies. One report has indicated that administration of Ad.p53 to glioma is hampered by the limited spread (<5 mm) of transduced cells (120). Both INGN 241 DNA and transgenic MDA-7/IL-24 protein distribute to a greater extent and demonstrate strong correlations with distance from injection site ($p<0.02$). Nucleic acid signals decay by 50% at 4-6-mm from the injection site whereas MDA-7/IL-24 protein and apoptosis signals decay by 50% at 18-20-mm. Therefore, either INGN 241 (Ad.*mda*-7) is more potent than other therapeutic constructs at inducing apoptosis in human tumors or the study design allowed us to capture signals that were lost in other studies. The levels of apoptosis induction observed in this study are substantially higher (average = 45%) than reported with other anticancer drugs. Apoptosis induction correlates with loss of Ki-67 staining (27). It is noteworthy that seven different tumor types were treated in cohorts 1-6 and all showed high levels of MDA-7/IL-24 expression and subsequent apoptosis induction. As discussed above, all of these patients had been heavily pretreated and failed multiple treatment regimens. It would be predicted that this group of chemo- and radio-resistant tumors would have acquired resistance to apoptosis. The fact that MDA-7/IL-24 induces apoptosis in such a spectrum of advanced tumor types mirrors preclinical studies and suggests that INGN 241 (Ad.*mda*-7) may have broad utility.

As discussed in other parts of this review, in addition to apoptosis induction of tumor cells and immunostimulation, additional 'bystander antitumor' activities have been reported for MDA-7/IL-24. Ad.*mda*-7 was reported to inhibit endothelial differentiation *in vitro*, implying antiangiogenic activity (24). Further studies demonstrated antiangiogenic activity of Ad.*mda*-7 *in vivo* (23), in that MDA-7/IL-24 expression significantly repressed angiogenic mediators, including VEGF, basic FGF, and IL-8. Studies using purified MDA-7/IL-24 demonstrated that it functions via the IL-22R1 on endothelial cells (92), and MDA-7/IL-24 was 50-fold more active than endostatin or angiostatin. When Ad.*mda*-7 was combined with irradiation, synergistic inhibition of tumor growth was observed, with significant reduction in microvessel density and pronounced apoptotic response in the tumor (23). Two recent preclinical studies have provided support for intriguing observations about the role of MDA-7/IL-24 in melanoma disease progression and metastasis. Initial studies found that MDA-7/IL-24 mRNA and protein were expressed in normal human melanocytes and benign nevi, but expression was lost in metastatic melanoma (2,11). Subsequent studies evaluated larger patient groups and concluded that MDA-7/IL-24 protein expression is progressively lost as melanoma tumors invade and become more metastatic and aggressive (10). These authors speculated that MDA-7/IL-24 must play a role in maintenance of normal physiology of melanocytes and that inhibition of MDA-7/IL-24 results in transformation and progression from a local, non-metastatic primary tumor to a highly metastatic phenotype (2,10,11). Additional studies have shown that Ad.*mda*-7 can inhibit cell migration and invasion of lung and breast tumor cells by down-regulating the PI3K pathway and inhibiting production of FAK and matrix metalloprotease (63,94). Tumor cells expressing MDA-7/IL-24 demonstrated significantly reduced lung metastases compared to control cells (76,94). It is clear that tumor cells can develop resistance to cytotoxic therapies, and reports are now emerging of acquisition of resistance to pathway-specific molecularly targeted therapeutics. The antitumor effects mediated by MDA-7/IL-24 encompass a variety of signaling pathways, and it is anticipated that redundant proapoptotic signals are activated (14). The ability of *mda*-7/IL-24 to enhance the therapeutic activity of radiation, monoclonal antibody therapy and chemotherapy provide additional avenues worth exploring as a means of enhancing the therapeutic index of this novel cytokine (5,6,14,16,18,26,35,37,38,40,42,58,61,62,65,68,118,121).

Transient increases in circulating cytokines such as IL-6, IL-10, and TNF-α in response to MDA-7/IL-24 were observed. Significantly higher elevations of IL-6 and TNF-α were

observed in patients receiving repeat treatment who demonstrated evidence of activity related to Ad.*mda*-7 (27). The majority of patients also showed a marked increase in $CD3^+$ and $CD8^+$ T cells at day 15 following injection, suggesting that Ad.*mda*-7 may be associated with a TH1 response. Furthermore, MDA-7/IL-24 has been shown to inhibit G_2/M cell cycling (12,24,50). The effect of MDA-7/IL-24 on tumor suppressor genes, such as ß-catenin and iNOS, was also evaluated (27). ß-catenin expression was reduced in six of nine patients and iNOS expression was reduced in four of nine patients tested.

In summary, Ad.*mda*-7 can induce a wide variety of effects associated with anticancer activity following intratumoral injection. Clinically significant responses were primarily seen with repeat injection. Future studies, therefore, will need to concentrate on repeat dosing of Ad.*mda*-7, particularly in malignant melanoma, in which the greatest clinical activity was seen in the first phase I study (29). Other future directions include developing systemic administration of Ad.*mda*-7, given its widespread tumor selectivity, exploration of its immunopotentiating effects, and its use in combinatorial strategies.

9. Future applications for improving the therapeutic index of *mda*-7/IL-24

In a remarkably short time frame, *mda*-7/IL-24 has gone from a laboratory discovery into the clinic. The results achieved to date using this gene are very encouraging and suggest that *mda*-7/IL-24 will provide significant benefit as a therapeutic for cancer. Although the results of the phase I clinical trial are provocative, it is clear that approaches to enhance the therapeutic benefit of *mda*-7/IL-24 are necessary to maximize its effectiveness in patients. Several approaches to achieve this objective were recently highlighted (14). One strategy, which has already proven efficacious, is the application of conditionally replication competent bipartite adenoviruses that both replicate and express *mda*-7/IL-24 in tumor cells (73,122, 123). These novel viruses have shown enhanced effectiveness in athymic nude mice containing human breast (73), colorectal (122) or therapy-resistant prostate (123) tumors in comparison with non-replicating adenoviruses expressing *mda*-7/IL-24. Augmentation of the antitumor properties of *mda*-7/IL-24 has also been achieved by using this gene in combination with radiation, chemotherapy or monoclonal antibody therapy (reviewed in refs. 18,19). Combining *mda*-7/IL-24 with agents that promote ROS production has been shown to augment therapeutic action of this gene in the context of prostate and pancreatic carcinomas (42,43). In this context, agents that can evoke this change in cancer cells may provide useful reagents to enhance therapeutic activity of *mda*-7/IL-24. Additionally, approaches that facilitate delivery of *mda*-7/IL-24 to the tumor microenvironment and tumor cells, including the use of stem cells and dendritic cells, offer promise for increasing therapeutic index of this novel cytokine (14). Although requiring further development and testing, modifying normal cells to produce and secrete MDA-7/IL-24 protein could in principle permit an even further expansion of clinical utility for this interesting molecule (14,35). Additionally, using agents that can induce MDA-7/IL-24 expression in cancer cells, thereby resulting in apoptosis, represents a unique therapy for cancer (124). Clearly, these and other approaches will require further study to define ways of more effectively using *mda*-7/IL-24 as a cancer gene therapeutic. We remain cautiously optimistic relative to the use and effectiveness of this novel cytokine for the therapy of diverse human malignancies.

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