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MDA-7/IL-24 Regulates Proliferation, Invasion and Tumor Cell Radiosensitivity: A New Cancer Therapy?

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Abstract The novel cytokine MDA-7/IL-24 was identified by subtractive hybridization in the mid-1990s as a cytokine whose expression increased during the induction of terminal differentiation, and that was either not expressed or was present at low levels in tumor cells compared to non-transformed cells. Multiple studies from several laboratories have subsequently demonstrated that expression of IL-24 in tumor cells, but not in non-transformed cells, causes their growth arrest and ultimately cell death. In addition, IL-24 has been noted to be a radiosensitizing cytokine, which in part is due to the generation of reactive oxygen species (ROS) and causing endoplasmic reticulum stress. Recent publications of Phase I trial data have shown that a recombinant adenovirus to express MDA-7/IL-24 (Ad.*mda-7* (INGN 241)) was safe and had tumoricidal effects in patients, which argues that IL-24 may have therapeutic value. This review describes what is known about the impact of IL-24 on tumor cell biology in addition to approaches that may enhance the toxicity of this novel cytokine. J. Cell. Biochem. 95: 712–719, 2005.

Key words: radiation; MDA-7; IL-24; kinase; caspase

MDA-7/IL-24: DISCOVERY AND CHARACTERIZATION

The gene for m*da-7* (recently renamed Interleukin 24, *IL*-24) was isolated from human melanoma cells induced to undergo terminal differentiation by treatment with interferon beta and mezerein [Jiang et al., 1995]. Based on sequence homology, MDA-7/IL-24 is classified as a member of the interleukin-10 (IL-10) family of cytokines, which includes IL-10, IL-19, IL-20, IL-22, and AK155 (IL-26) [Jiang et al., 1995; Goris et al., 2001; Caudell et al., 2002; Ellerhorst et al., 2002]. It was noted that IL-24 protein expression is decreased in advanced melanomas [Jiang et al., 1995; Ellerhorst et al., 2002], with nearly undetectable levels in metastatic disease, in general agreement with this gene product being classified as a tumor suppressor [Ekmekcioglu et al., 2001; Lebedeva et al., 2002]. Other manuscripts over the last 8 years have demonstrated that enforced expression of IL-24, either by transfection of a plasmid containing the cDNA for *mda-7*/*IL-24* or by use

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of a recombinant adenovirus, Ad.*mda-7*, rapidly inhibits the growth of a broad spectrum of cancer cells, resulting in tumor cell death within 24–48 h [Jiang et al., 1996; Su et al., 1998]. IL-24 was also noted to be a secreted [Su et al., 2001; Sauane et al., 2003a]. Of considerable note, when IL-24 was expressed in non-transformed cells in vitro, such as normal human epithelial or fibroblastic cells, little modification was observed in either cell growth or cell viability [Jiang et al., 1996; Su et al., 1998; Huang et al., 2001; Mhashilkar et al., 2001; Ellerhorst et al., 2002] (Figs. 1 and 2).

Despite the in vitro evidence noted above, where viral expression of IL-24 caused tumor cell-specific cell killing, the biological anti-tumor actions of IL-24 have also been noted in vitro and in vivo to depend on both its anti-migratory and anti-differentiation actions on vascular endothelial cells. IL-24 prevents VEGF- and bFGF-induced endothelial tube formation in vitro and angiogenesis in vivo [Ramesh et al., 2003, 2004]. More recent studies have also argued that IL-24 radiosensitizes non-transformed human vascular endothelial cells, which would represent the first instance where IL-24 has been shown to reduce cell survival in a non-transformed cell type. Thus IL-24 is a potent anti-angiogenic cytokine [Nishikawa et al., 2004].

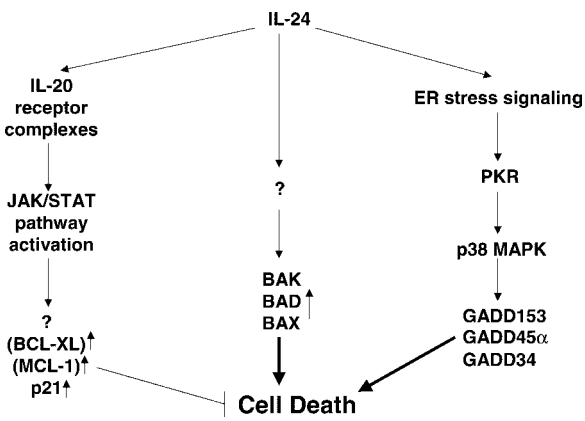

Fig. 1. Mechanisms by which IL-24 may cause cell death [Yacoub et al., 2004; Lebedeva et al., 2005]. IL-24 was initially noted to promote cell death by activating the p38 MAPK pathway and promoting cell death via GADD transcription factors. Activation of p38 MAPK may in part be due to IL-24-dependent ER stress and the activation of PKR. IL-24 can inhibit and activate ERK1/2 signaling, which plays a cell type dependent role in either promoting or inhibiting cell death. In other studies, IL-24 has been noted to increase expression of TRAIL (extrinsic pathway), BAX (intrinsic pathway), BAD (intrinsic pathway) and BAK (intrinsic pathway).

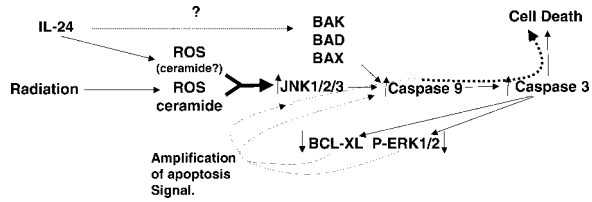

Fig. 2. Mechanisms by which IL-24 may promote radiosensitization. Based on the cell type, IL-24 and radiation either individually have been shown to promote activation of the JNK1/2/3 pathway or have combined together to promote JNK1/2/3 activation. The mechanisms of JNK1/2/3 pathway activation are unclear, for example, ceramide generation, but definitely include the generation of reactive oxygen species (ROS). Activation of JNK1/2/3 is essential for mitochondrial dysfunction and apoptosis. In some cell types, a loss of anti-apoptotic protein expression and a loss of ERK1/2 phosphorylation are secondary events that occur after the primary activation of JNK1/2/3 and the initiation of caspase activation.

The pathways by which Ad.*mda-7* (or transfection with a cDNA to express IL-24) enhances apoptosis in tumor cells are not fully understood, however, evidence from several studies suggests the involvement of proteins important for the onset of growth inhibition and apoptosis via the intrinsic/mitochondrial apoptotic cell death pathway including BCL-XL, BCL-2, and BAX [Su et al., 1998; Mhashilkar et al., 2001; Lebedeva et al., 2003a; Sauane et al., 2003a]. In melanoma cell lines, but not in normal melanocytes, infected by Ad.*mda-7* it was noted that a significant decrease in both BCL-2 and BCL-XL levels occurred, with a more modest up-regulation of BAX and BAK expression [Lebedeva et al., 2002]. This data supports a hypothesis that Ad.*mda-7* enhances the ratio of pro-apoptotic to anti-apoptotic proteins in cancer cells, thereby facilitating induction of apoptosis [Su et al., 1998; Madireddi et al., 2000; Lebedeva et al., 2002; Sauane et al., 2003a,b; Pestka et al., 2004]. The ability of Ad.*mda*-7 to induce apoptosis in the prostate cancer cell line, DU145, which does not produce BAX, indicates that IL-24 can mediate apoptosis in tumor cells by a BAX independent pathway [Madireddi et al., 2000; Saeki et al., 2000; Sarkar et al., 2002a; Sauane et al., 2003a; Lebedeva et al., 2003b].

In addition to modification of mitochondrial function, IL-24 has also been argued to kill cancer cells by causing endoplasmic reticulum stress as well as by enhancing death receptor signaling. Studies by [Pataer et al., 2002] demonstrated that IL-24 promoted activation of PKR, which was correlated to enhanced eIF2

alpha phosphorylation and IL-24-stimulated cell death. In this study PKR null fibroblasts were resistant to IL-24-induced apoptosis, although a subsequent study from the same group has argued PKR does not play a role in the lethal effects of IL-24 [Chada et al., 2004; Nishikawa et al., 2004]. Unpublished studies from this group have recently argued that transformed PERK −/− MEFs are more sensitive to apoptosis induced by GST-IL-24 than wild type MEFs (Koumenis, Yacoub, Gupta, Fisher, and Dent, unpublished observations). One report has argued that activation of death receptor signaling is also partly responsible for IL-24 stimulated apoptosis [Saeki et al., 2002]. It has also been noted that Ad.*mda-7* toxicity occurs in tumor cells that do not express IL-20 receptor complexes, arguing that activation of these receptors, and potentially STAT transcription factors, is dispensable for IL-24 lethality [Sauane et al., 2003b, 2004a; Nishikawa et al., 2004]. Collectively, these findings argue that IL-24, when introduced into cells as a cDNA, in a recombinant adenovirus, or as a purified protein (see also below) promotes cancer cell-specific cell death in multiple tumor cell types and by multiple apoptotic mechanisms in an IL-20 receptor-independent fashion.

MDA-7/IL-24: CHARACTERIZATION OF PURIFIED IL-24 AND GST-IL-24 PROTEINS

Use of purified IL-24 protein (synthesized in either bacteria as a GST fusion protein or in mammalian cells as a FLAG or $(His)_6$-tagged protein) has shown variable effects on the growth, migration, and viability of transformed and non-transformed cells [Su et al., 2003; Sauane et al., 2004b]. Initial studies using mammalian cell synthesized IL-24 protein in cells demonstrated that purified IL-24 interacted with two type II cytokine hetero-dimeric receptor complexes: IL-20R1/IL-20R2 (type 1 IL-20R) and IL-22R1/IL-20R2 (type 2 IL-20R) [Dumoutier et al., 2001; Wang et al., 2002]. In one of the first of these studies, non-transformed BHK cells stably transfected with IL-20 and IL-22 receptors were treated with MDA-7/IL-24; at low pM concentrations of IL-24 (<100 pM) growth was promoted whereas at higher concentrations (>100 pM) IL-24 inhibited cell proliferation [Parrish-Novak et al., 2002]. In transfected cells, IL-24 activated multiple STAT transcription factors. However, in OVCAR-3 ovarian carcinoma cells, which express endogenous IL-20 receptor complexes, it was noted that IL-24 at low nM concentrations promoted growth inhibition without altering STAT transcription factor phosphorylation/function [Parrish-Novak et al., 2002]. Other studies have demonstrated using tumor cells, which lack STAT1 or STAT3 function or with blocked Janus kinase function that STAT pathway signaling is not required for IL-24-induced growth arrest or tumor cell killing [Sauane et al., 2003b; Nishikawa et al., 2004].

More recent studies have indicated a difference in the cell signaling and cell killing properties of bacterial synthesized GST-IL-24 and mammalian cell synthesized IL-24 with FLAG or $(His)_6$ tags to aid isolation. In multiple studies using a wide variety of transformed cell lines, GST-IL-24 has been noted to promote cell growth arrest and apoptosis in a tumor cell-specific fashion. Furthermore, GST-IL-24 has been noted to cause these effects independently of expression of IL-20 receptors, in a similar manner to Ad.*mda-7* [Sauane et al., 2003b, 2004]. This would suggest that GST-IL-24 is taken up by cancer cells in an interleukin-receptor independent fashion. In contrast to GST-IL-24 and Ad.*mda-7*, purified IL-24, synthesized in mammalian cells, does not appear to have any biologic effect on cells lacking expression of the IL-20 receptor complexes [Nishikawa et al., 2004]. Of note however, and in a similar manner to GST-IL-24 and Ad.*mda-7*, in cells where IL-20 receptor complexes were expressed, mammalian synthesized IL-24-induced cell killing was independent of STAT transcription factor activation. For example, in A549 human lung carcinoma cells, which lack expression of the IL-20 receptor complexes, mammalian synthesized IL-24 has no biologic effect on cell growth/viability, whereas GST-IL-24, Ad.*mda-7*, Ad.*mda-7*$^{SP-}$, which expressed a non-secreted form of IL-24 and transfection of a plasmid to express IL-24 all promote growth arrest and cell death [Su, Emdad, Sauane, Lebedeva, Sarkar, Gupta, James, Randolph, Valerie, Walter, Dent, and Fisher, unpublished observations]. Furthermore, whilst it has been noted that IL-24, IL-20, and IL-19 all activated STAT transcription factors in IL-20 receptor expressing cancer cells, only IL-24 has the capability to cause cell death. Collectively, this data argues that the tumoricidal effects of IL-24

are independent of IL-20 receptor complex signaling and instead dependent on an additional poorly defined biological property of IL-24. Whether the receptor binding domain of IL-24 or other portions of the molecule plays a role in this interleukin receptor-independent tumoricidal biological function of IL-24 has yet to be determined.

MDA-7/IL-24: THE INTERACTION OF IL-24 WITH AGENTS THAT GENERATE REACTIVE OXYGEN SPECIES

Based on the findings of many laboratories using a diverse set of therapeutic agents, it would be expected that IL-24 as a single agent could have limited potential in a patient to treat cancer, particularly a patient with metastatic disease, implying that IL-24 therapy would need to be combined with other therapeutic modalities to achieve an improved clinical response. Encouraging recent data from Phase I trails has argued that injection of patient tissue and tumors in situ with Ad.*mda-7* causes measurable tumor cell death in vivo, both in infected tumor cells and in uninfected tumor cells many centimeters away from the site of injection, implying that IL-24 is expressed and secreted from infected cells, and has a so called apoptotic "bystander effect" on distant uninfected tumor cells [Fisher et al., 2003; Cunningham et al., 2005; Lebedeva et al., 2005; Tong et al., 2005]. Clearly, however, at sites more distant to viral administration where IL-24 concentrations are only growth inhibitory and not cytotoxic, the combination of IL-24 therapy with established therapeutic agents to enhance the toxicity of IL-24 would be of considerable clinical utility.

Ionizing radiation causes ionizing events in water, generating hydroxyl radicals that can impact on the function of mitochondria in cells, which in turn amplify the initial free radical signaling, generating large amounts of reactive oxygen and nitrogen species [Leach et al., 2001, 2002]. In addition, radiation can cause DNA damage, activate poly ADP ribosyl polymerase (PARP) leading to an altered cellular redox status, which can also be sensed by mitochondria. Radiotherapy is used as a primary modality for the treatment of many malignancies including those of the breast, brain, prostate, and lung. Based on the tumoricidal effects of both radiation and IL-24, it was a logical step for investigators to determine whether IL-24 had radiosensitizing potential. Several laboratories have demonstrated that Ad.*mda-7*, GST-IL-24 and IL-24 can radiosensitize a wide variety of tumor cell lines in vitro and in vivo [Su et al., 2003; Yacoub et al., 2003a, 2004; Nishikawa et al., 2004]. In studies using human glioma and prostate carcinoma cells, the ability of both ionizing radiation and IL-24 to generate reactive oxygen species (ROS) was directly linked to the radiosensitizing properties of IL-24 [Yacoub et al., 2003a,b; Lebedeva et al., 2003b, 2005]. Other therapeutic agents have also been shown to act, in part, by generating ROS, including arsenic trioxide and 4-hydroxyphenyl-retinamide (4-HPR) [Lebedeva et al., 2003b, 2005; Yacoub et al., 2003c]. In general agreement with ROS enhancing the lethal actions of IL-24, combined treatment of renal, pancreatic and prostate carcinoma cells with Ad.*mda-7* or GST-IL-24 and arsenic trioxide or 4-HPR resulted in a highly synergistic potentiation of tumor cell killing that was not manifested in non-transformed renal or prostatic epithelial cells [Lebedeva et al., 2003b, 2005; Yacoub et al., 2003b]. Collectively, these findings argue that established and novel therapeutic modalities, which generate ROS can promote IL-24 lethality in cancer cells.

MDA-7/IL-24: THE REGULATION OF SIGNAL TRANSDUCTION PATHWAYS AND THEIR ROLE IN MODULATING CELL GROWTH AND SURVIVAL AFTER CYTOKINE EXPOSURE

The regulation of signal transduction pathway functions by Ad.*mda-7* and IL-24 protein, particularly when combined with ionizing radiation, appears to be as complicated as the number of mechanisms by which IL-24 has been reported to induce cell death. As noted previously, activation of STAT transcription factors does not appear to significantly modulate IL-24 lethality, despite IL-24 activating STAT transcription factors through IL-20 receptor complexes. However, data in several tumor cell types has argued that either Ad.*mda-7* or IL-24 protein promote activation of the p38 mitogen activated protein kinase (MAPK) pathway, which via GADD34 promotes cell death [Sarkar et al., 2002b; Su et al., 2003]. In part, this may be explained by data suggesting IL-24 causes PKR activation in some tumor cell types, which is a known up-stream activator of both p38 MAPK

and GADD34 [Pataer et al., 2002; Silva et al., 2004]. Surprisingly, as a single agent, IL-24 has not been noted to significantly modulate cell death via the JNK1/2/3 pathway (see also below). Other studies have also argued that IL-24 modulates PI3K/AKT and ERK1/2 pathway function, which in the case of ERK1/2 signaling, in a cell type dependent manner, can either protect from IL-24 lethality or promote IL-24-dependent apoptosis [Mhashilkar et al., 2003; Yacoub et al., 2003b, 2004]. Thus an overriding concept of IL-24 promoting cell death by activating "one pathway" and inhibiting "another pathway," as has been argued for other therapeutic agents, has only partially evolved. An additional essential point to make with all of the above published studies, particularly those using Ad.*mda-7*, is that much of the signaling data have been generated at the same time as apoptosis analyses were performed, that is, the initial signaling responses of cells to IL-24 treatment/expression, prior to the induction of cell cycle arrest and cell killing, have not been fully determined. Clearly, additional studies will be required to understand the regulation of signaling pathway function by IL-24.

As a single agent ionizing radiation induced cell killing in a variety of cancer cells has been linked to the activation of the c-Jun NH_2-terminal kinase (JNK) and in certain cell types the p38 MAPK pathway [Dent et al., 1999; Vrana et al., 1999; Cartee et al., 2000]. When combined with ionizing radiation, IL-24 has been argued to promote radiation toxicity by modulating JNK1/2/3 pathway signaling, and to a lesser extent activation of p38 MAPK. For example, [Kawabe et al., 2002] demonstrated that lung cancer cells were radiosensitized by Ad.*mda-7* via JNK1/2 signaling, without radiation further enhancing IL-24-induced JNK1/2 activation. [Yacoub et al., 2003a] using established rodent and human glioma cell lines, as well as primary human glioma cell isolates, demonstrated that Ad.*mda-7* caused radiosensitization in vitro and in vivo, and that in vitro sensitization was dependent, in part, on JNK1/2/3 activation [Yacoub et al., 2003b, 2004]. In these studies, radiation produced a rapid and transient (~6 h) increase in JNK1/2/3 activity, which had dissipated to near basal levels 48–96 h after exposure. In established and primary GBM cells, Ad.*mda-7* or GST-IL-24 weakly enhanced JNK1/2/3 phosphorylation over 96 h of infection/treatment. However, combined treatment of cells with radiation and IL-24 resulted in a prolonged intense activation of JNK1/2/3. Many groups have argued that prolonged intense JNK1/2/3 pathway signaling is involved in cell death processes. In agreement with this concept, inhibition of JNK1/2/3 signaling abrogated the radiosensitizing effect of IL-24 in glioma cells.

As mentioned previously, studies in primary glioma cells have also demonstrated that a careful consideration of the primary and secondary effects of IL-24 on cell signaling and the expression of pro-/anti-apoptotic effector proteins is required by investigators. For example, as a single agent, low concentrations of GST-IL-24 that were marginally toxic promoted activation of ERK1/2 and enhanced expression of the pro-apoptotic molecules BAX and BAD [Yacoub et al., 2004]. The combination of GST-IL-24 and radiation reduced ERK1/2 phosphorylation, reduced BCL-XL expression and further enhanced BAX and BAD levels. However, the effects on ERK1/2 phosphorylation and BCL-XL expression were not primary effects due to either IL-24 treatment or radiation exposure, but were instead secondary effects dependent on prior induction of apoptotic caspase activities by the combination of IL-24 and radiation. With regard to these findings, it is of note that several studies in other cell types have directly linked JNK1/2/3 pathway activation to mitochondrial dysfunction and the promotion of apoptosis, including phosphorylation and inactivation of the anti-apoptotic molecules BCL-2 and BCL-XL [Fan et al., 2000; Basu and Haldar, 2003].

Studies with other agents that generate ROS have also linked activation of the JNK1/2/3 and p38 MAPK pathways to the potentiation of IL-24 toxicity. In renal carcinoma cells GST-IL-24 was noted to interact with arsenic trioxide and 4-HPR to promote activation of JNK1/2 and p38 MAPK, which appear to both influence the potentiation of cell killing [Yacoub et al., 2003a]. In pancreatic carcinoma cells, Ad.*mda-7* and agents that generate ROS differentially modified signaling pathway activities, even in cells with very similar genetic backgrounds, for example, mutated active K-RAS and mutated p53 [Lebedeva et al., 2005]. Nevertheless, Ad.*mda-7* and agents that generate ROS all interacted to promote pancreatic carcinoma cell killing. Thus even within tumor cells derived from the same organ, it is possible for IL-24 to promote cell killing by modulating the activities

of multiple different signal transduction pathways in a cell-type specific manner.

Finally, as mentioned in several sections, IL-24 has been noted to promote increased expression of pro-apoptotic BH3 domain containing proteins such as BAX, BAK, and BAD. Combined exposure of carcinoma cells to ROS generating agents and IL-24 can further increase the expression of such pro-apoptotic molecules. However, unlike studies examining the JNK1/2/3 and ERK1/2 signal transduction pathways, IL-24 appears to promote enhanced expression of pro-apoptotic BH3 domain containing proteins in an ROS-independent fashion [Yacoub et al., 2004]. Future studies will be required to understand how IL-24 increases expression of BH3 domain proteins.

MDA-7/IL-24: CONCLUSIONS

Studies by multiple laboratories have demonstrated that IL-24 represents a cytokine with potent anti-tumor properties, in vitro, in vivo and in Phase I patient trials. These properties are dependent upon at present poorly defined, or at the very least cell type specific, signaling pathways that lead to growth arrest and cell death. In addition to direct effects on tumor cells, IL-24 has also demonstrated profound anti-angiogenic properties. IL-24, even at low concentrations, which by themselves are not toxic, has been shown to be a potent radiosensitizer. Collectively, these findings strongly argue that IL-24 has considerable potential as a cancer therapeutic agent.

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