of MKK, MAPK, and AP-1. These observations indicate that Raf-1 is an effector of Ras in IR-stimulated MAPK pathway.

Previously, we have demonstrated a correlation between antisense *c-raf 1* cDNA mediated inhibition of Raf-1, delayed tumor growth, and enhanced radiosensitivity of relatively radioresistant laryngeal squamous carcinoma cells (SQ-20B). Furthermore, *c-raf-1* cDNA transfection results in a relatively radioresistant phenotype of human immortalized bronchial epithelial cells and human squamous carcinoma cells. Consistent with these data, antisense *raf* oligodeoxyribonucleotide (AS-*raf*-ODN)-specific inhibition of Raf-1 protein has been associated with radio sensitization of SQ-20B tumor cells. These and other studies utilizing mouse embryo fibroblasts containing targeted disruption of the c-raf-1 gene suggest that Raf-1 is an important cell survival factor.

Translation of laboratory observations into clinically relevant protocols is an important step towards the ultimate goal of developing safer and more effective cancer therapies. Along these lines, novel cationic liposomes have been designed to systemically deliver AS-*raf*-ODN to tumor tissues. Safety and pharmacokinetics profile of liposomes carrying AS-*raf*-ODN have been established in different animal species. It should be noted that unmodified AS-*raf*-ODN is nontoxic *in vivo* and cationic liposomal composition that we designed is also non-toxic. Plasma and normal tissue pharmacokinetics of liposome-encapsulated unmodified AS-*raf*-ODN (LE-AS-*raf*-ODN) is significantly better compared to "free" AS-*raf*-ODN. In addition, systemic delivery of the liposomal formulation of a phosphorothioated AS-*raf*-ODN (LE-5132) provides an improved tumor control compared to "free" phosphorothioated AS-*raf*-ODN (5132) ($P < 0.001$). Intravenous administration of LE-5132 leads to radiosensitization of SQ-20B tumor xenografts. Histopathologic evaluation of these tumor specimens revealed a significant proportion of cells containing fragmented chromatin compared to single agent treated or control groups. These data demonstrate that AS-*raf*-ODN is an effective radiosensitizer *in vivo*.

To identify new cellular targets of cancer gene therapy, we have used the differential gene expression technology and selected for genes aberrantly expressed in metastatic or radiation-resistant tumor cells but not in the matched primary tumor-derived cells or radiosensitive tumor cells. In addition, candidate genes downstream of Raf-1 have been identified using AS-*raf*-ODN and comparing the gene expression patterns in isogenic tumor cell populations expressing differential steady state levels of Raf-1. Because AS-*raf*-ODN treatment of a variety of human tumor cells causes apoptosis, characterization of novel effectors downstream of Raf-1 should provide further insight into the mechanisms regulating cell death or cell survival.

In conclusion, these studies establish an important role of Raf-1 in radiation resistance and radiation-initiated intracellular signal transduction process. In addition, our data provide a basis for a clinical study of the combination of AS-*raf*-ODN and radiation in cancer patients.

In Vivo Protection Against Gamma-Irradiation with 5-Androstenediol

MARK H. WHITNALL, THOMAS B. ELLIOTT, MICHAEL R. LANDAUER, WILLIAM E. JACKSON III, CATHERINE L. WILHELMSEN, LUANN MCKINNEY, K. SREE KUMAR, VENKATARAMAN SRINIVASAN, G. DAVID LEDNEY, AND THOMAS M. SEED

Radiation Casualty Management Team, Armed Forces Radiobiology Research Institute, Bethesda, MD 20889-5603

5-androstenediol (AED) is a representative of a novel class of radioprotectants, the 5-androstene steroids, that enhance immune function and promote survival after whole-body exposure to ionizing radiation. Improvements in survival were observed when AED was administered by sc injection between 24 h before and 2 h after gamma-irradiation of mice. A dose reduction factor of 1.3 was calculated from probit survival curves. Protection was observed in both male and female mice, with and without subsequent inoculation with lethal doses of *Klebsiella pneumoniae*. No protection was observed with a number of other steroids: dehydroepiandrosterone (DHEA), 5-androstene-3B,7B, 17B-triol (AET), androstenedione, or estradiol. AED induced increases in circulating neutrophils, platelets, and NK cells in normal or irradiated mice, with no effect on numbers of circulating B cells or T cells. Numbers of granulocyte-monocyte progenitors in bone marrow were also increased with AED. No signs of toxicity were observed in

clinical chemistry, histopathology, or behavioral assays. CD11b expression on circulating NK cells was markedly increased by whole body irradiation and pretreatment with AED in a synergistic manner. CD11b expression on circulating neutrophils and monocytes was unchanged or slightly decreased. The efficacy and low toxicity of AED make it an attractive candidate for development as a countermeasure for the injurious effects of ionizing radiation.

Acute exposure to high doses of ionizing radiation results in failure of hemopoietic progenitors to proliferate, causing mortality due to infection and hemorrhage. Pharmacologic countermeasures include free radical scavengers and antioxidants that serve to minimize initial injury, as well as immunomodulators and cytokines that can stimulate hemopoietic recovery. Since prophylaxis with DHEA and its metabolite AED increase survival during bacterial and viral infections in unirradiated animals, we have been investigating the use of this family of steroids as a countermeasure to whole body gamma-irradiation at doses that induce mortality due to decreased resistance to infection.

Materials and Methods

Male CD2F1 and female B6D2F1 mice (Jackson) were maintained and irradiated at 0.6 Gy/min in the AFRRI 60 Co facility as described. AED (Steraloids) was dissolved in PEG-400 vehicle (Sigma). Clonal assays for marrow granulocyte-monocyte progenitors (GM-CFC) were performed as described. In some experiments, AED was compared to DHEA, 5-androstene-3B,7B, 17B-triol (AET), androstenedione or estradiol (all from Steraloids). CDIIb surface expression on circulating neutrophils was measured using a monoclonal antibody (Pharm-ingen) and a FACSCalibur flow cytometer. Blood cell counts were performed on an Advia 120 (Bayer). Clinical chemistry of serum was analyzed using an Ektachem-700 (Kodak). Histopathology was performed using H&E-stained paraffin sections prepared and examined by standard methods. Behavioral analysis was carried out using an inverted screen test for motor coordination, a strain gauge for forelimb grip strength, and a computerized activity monitor (Omnitech) for locomotor activity.

Results and Discussion

We injected AED sc at a dose of 160 mg/kg into male CD2F1I or female B6D2F1 mice 24 h before whole-body gamma irradiation to calculate dose reduction factors (DRI's, the amount by which a probit survival curve is shifted by treatment). The doses of radiation received ranged between 7 and 12 Gy, and the DRIs for male and female mice were 1.23 and 1.26, respectively, indicating that the survival-enhancing effects of AED are not specific for strain or gender. In addition, female B6D2F1 mice were injected with AED, exposed to radiation at doses of 3 to 7 Gy, and inoculated with lethal doses of *Klebsiella pneumoriae* 4 days later, at the nadir of circulating white blood cells. The resulting DRF was 1.18. In separate experiments, survival was enhanced when AED was injected 1 to 2 h after irradiation, in K pneumoniae inoculated mice.

When similar experiments were done comparing AED to DHEA, AET, androstenedione or estradiol, the other steroids exhibited no significant protective effects on survival. The data indicate that the survival-enhancing effects of AED are due to interactions with specific receptors. This conclusion is supported by the fact that a significant dose-response curve was obtained when AED was injected sc at 40–320 mg/kg and circulating neutrophils and platelets were measured 4 and 14 days later, respectively.

AED given 24 h before irradiation (3 Gy) significantly ameliorated the radiation-induced neutropenia and thrombocytopenia. The effects on numbers of circulating neutrophils were due to AED-induced stimulation of myelopoiesis, as indicated by amelioration of radiation induced decreases in marrow GM-CFC. Furthen-nore, AED caused increases in marrow myeloid progenitors in unirradiated mice.

AED also caused increases in circulating monocytes and NK cells in both irradiated and unirradiated mice, but had no effect on numbers of circulating T cells or B cells, indicating that the primary effect of AED is on the innate rather than the adaptive immune system. CD11b measured by flow cytometry showed small decreases or no changes on neutrophils or monocytes in response to AED, while AED and whole-body irradiation together increased levels of surface CDIIb on NK cells. The data indicate synergistic activation of NK cells by AED and radiation.

The beneficial effects of AED are significant, and are not accompanied by significant toxic effects, as shown by clinical chemistry, histopathology, or behavioral assays.

We conclude that AED is the first in a novel class of 5-androstene steroid radioprotectants, enhances survival and hemopoiesis after irradiation, and is non-toxic. These attributes, together with the facts that these compounds are stable during storage at high temperatures and have anticancer properties, make them attractive candidates for further exploration as countermeasures against the short- and long-term effects of ionizing radiation.

The authors thank Cynthia Inal, Venita Miner, Rita Harding, Vilmar Villa, Karen Quinones, Brian Hively, Jason Deen, Sharneek Ghose, Sarny Bouhaouala, Robin-Anne Ferris, Lennox Jobe, Ray Toles, Jeya Yogaraj, Lillie Hernan-Ackah, Jon Findlay, John Ferrell, Alan Chap, Michael Wang, and Peter Wang for excellent technical assistance.

1. Hendry JH, Feng-Tong Y. Response of bone marrow to low LET irradiation. In: Hendry JH, Lord BI, Eds. Radiation Toxicology: Bone Marrow and Leukaemia. London: Taylor & Francis: pp91–116, 1995.
2. Bump EA, Malaker K, Ed. Radioprotectors. Chemical, Biological and Clinical Perspectives. Boca Raton, FL: CRC Press, 1998.
3. Loria RM, Padgett DA. Androstenediol regulates systemic resistance against lethal infections in mice Arch Virol **127**:103–115, 1992.
4. Whitnall MH, Elliott TB, Harding RA, Inal CE, Landauer MR,

Wilhelmsen CL, McKinney L, Miner VL, Jackson WE, Loria RM, Ledney GD, Seed TM. Androstenediol stimulates myelopoiesis and enhances resistance to infection in gamma-irradiated mice. Int J Immunopharmacol **22**:1–14, 2000.
5. Landauer MR, McChesney DG, Ledney GD. Synthetic trehalose dicorynomycolate (S-TDCM): behavioral effects and radioprotection. J Radiat Res (Tokyo) **38**:45–54, 1997.
6. Schwartz AG, Pashko LL. Cancer prevention with dehydroepiandrosterone and non-androgenic structural analogs. J Cell Biochem Suppl **22**:210–217, 1995.

Chelated Metal Ions for Therapeutic and Diagnostic Applications

MARTIN W. BRECHBIEL
Radioimmune & Inorganic Chemistry Section, DCS, NCI, NIH, Bethesda, MD, 20892

Use of radiolabeled monoclonal antibodies (mAb's) that localize and bind to malignant cells continues to be an attractive mechanism for targeting and delivering either an imagable isotope, be it a γ-emitter or B+-emitter, or a particle emitter such as a B−-emitter or an a-emitter for therapeutic applications.

Mab's can be viewed as macromolecular targeting reagents that possess: a discrete chemical structure, sites for chemical modification, and can be made available in high purity and in workable amounts. For these applications, there are minimal criteria that must be met to insure success. The methods employed to radiolabel mAb's must not alter specificity nor alter the rate of catabolism. Finally, of paramount importance, the radiolabel must be securely linked to the mAb. Conjugation to mAb of a radio-metal complex via suitable bifunctional chelating agents provides a wide variety of possible half-lives and emission characteristics. The complex then must be adequately thermodynamically and kinetically stable to minimize release of the isotope *in vivo*.

Numerous recent reports of positive results from clinical trials involving the use of B−-emitting radiolabeled antibodies and the majority have used either 131I (t1/2 = 8.04 d) and 90Y (t1/2 = 64.1 h) for the treatment of either leukemias or lymphomas. These results validate the potential for this modality being a viable clinical therapy. Despite these results, the range of the B− particle is several millimeters and the majority of energy deposition does not occur immediately along the emission track. Cytotoxicity attributed to mAb's radiolabeled with 90Y has been attributed to result from "cross-fire" and not as a direct effect from the emission occurring at the cell surface. Therefore, B−-emitting radioisotopes have been proposed to be less useful for micrometastatic disease and thus might also contribute to normal tissue toxicity. An a-emitting radionuclide might be a better choice in this case due to very high cytotoxicity, a short path length emission, and immediate energy deposition that should minimize collateral cytotoxicity. Examples of metallic-emitters that have been studied are 212 Bi (t1/2 = 45 min) and 213 Bi (t1/2 = 45 min).

Bifunctional derivatives of diethylenetriamine pentaacetic acid (DTPA) developed for use with 90Y proved to form complexes with Bi(III) that were labile *in vivo*. Efforts to develop improved ligands for 90Y that incorporated a trans-cyclohexyl ring into the backbone of DTPA led to the family of CHX-DTPA ligands, which were found to meet the requisite criteria for use of the Bi(III) isotopes. Preclinical experiments confirmed both the stability of the CHX-DTPA ligands for the Bi(III) isotopes and the therapeutic applicability of these cc-emitting isotopes such that a phase I clinical trial was initiated to treat AML at Memorial Sloan-Kettering with an antiCD33 antibody using 213 Bi.

Objectives and Results

Many have expounded that use of 213 Bi might be limited by its half-life to treatment of circulatory malignancies. Despite this, we chose to investigate the possibility of using an engineered mAb, HuCC49CH2, which had been reported to target very rapidly while exhibiting efficient whole body clearance. An initial experiment performed was to determine a maximum tolerated dose (MTD) for the 213 Bi labeled immunoconjugate. As there was minimal information concerning the use of a combination of this mAb, 213Bi, and ip administration, four dose ranges were used in 30 mice that had been inoculated with an LS- I 74T tumor 10 days prior to treatment (50–200 mm3). Results ranged from no response (80–115 uCi), delayed tumor growth (149 uCi, 223–270 uCi), to significant regression of tumor growth with the high dose (316–450 uCi) with no overt toxicity observed in any animal. The experiment was repeated to determine the MTD, but also to verify that this response was valid and whether it could be expanded. To this end, mice were again inoculated with tumors in the flank (83.8 more or less of 31.5 mm3) and four different doses were again administered ip. All of the controls (n = 5) had to be terminated after 14 days due to tumor size. At