Elevated Epidermal Growth Factor Receptor Binding in Plutonium-Induced Lung Tumors from Dogs (43203)

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Abstract. **The objective of this study is to examine and characterize epidermal growth factor receptor (EGF-R) binding in inhaled plutonium-induced canine lung-tumor tissue and to compare it with that in normal canine lung tissue. Crude membrane preparations from normal and lung-tumor tissue from beagle dogs were examined in a radioreceptor assay, usng ^{125}I-labeled epidermal growth factor (EGF) as a ligand. Specific EGF receptor binding was determined in the presence of excess unlabeled EGF. We have examined EGF receptor binding in eight lung-tumor samples obtained from six dogs. Epidermal growth factor receptor binding was significantly greater in lung-tumor samples (31.38%) compared with that in normal lung tissue (3.76%). Scatchard plot analysis from the displacement assay revealed that there was no statistical difference in the binding affinity but significantly higher concentration of EGF-R sites in the lung-tumor tissue (619 fmol/mg) than in normal lung tissue (53 fmol/mg). The increase in EGF-R number in plutonium-induced dog lung tumors does not seem to correlate with increase in the initial lung burden exposure to plutonium. Our results demonstrate that there is a significant increase in EGF-R binding in inhaled plutonium-induced dog lung tumors.**
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The dose-effect relationships of inhaled plutonium in dogs are being studied to help evaluate the health risks of accidental human exposure (1). To establish the animal models as accurate predictors of human risk in toxicity studies, it is important to use routes of exposure and demonstrate metabolism of toxic materials that are similar to those for humans, and to demonstrate a similar molecular pathogenesis of developing lesions in animals and humans. It has been established that the inhalation of plutonium by beagle dogs produces lung tumors, but the molecular mechanisms of radiation oncogenesis have not been demonstrated.

Epidermal growth factor (EGF) is a polypeptide that regulates the growth and differentiation of a great variety of cells through a specific EGF receptor (EGF-R) (2, 3), and it is well established that the EGF and EGF-R growth regulatory system is involved in both normal and neoplastic cellular growth and differentiation (4, 5). Recently, enhanced EGF-R levels have been reported in human lung cancer and in human lung cancer cell lines, as shown by the radioreceptor assay and by immunocytochemical assay (6–11). The abnormally high expression of EGF-R is primarily associated with non-small-cell carcinoma of the human lung (6, 8). However, there are no experimental data demonstrating whether growth factors and their receptors are abnormally expressed in animal lung tumors. Since radiation-induced dog lung tumors are mainly non-small-cell carcinoma, we decided to examine the EGF-R in plutonium-induced dog lung tumors by radioreceptor binding assay.

Materials and Methods

Dog Lung Tissues. Primary lung-tumor tissues were obtained from five dogs exposed to inhaled plutonium and from one control dog from our ongoing long-term experiments to determine the lifespan dose-effect relationships of inhaled plutonium (1) (Table I). In addition, normal lung tissue was obtained from two dogs with lung tumors and from 13 additional, tumor-

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free "control" dogs. These specimens were immediately frozen and stored at −70°C until assayed.

Radiorecptor Assay. Mouse EGF, obtained from Collaborative Research, Bedford, MA, was iodinated by a modified lactoperoxidase method similar to that of Thorell and Johansson (12), as described by Leung (13). Briefly, the H_2O_2 was added in four or five aliquots at 1-min intervals to a reaction mixture of 1 mCi of ^{125}I, 10 μg of EGF, and 10 μg of lactoperoxidase (bovine milk, obtained from Calbiochem, La Jolla, CA). The labeled EGF was separated from the free [^{125}I]NaI and lactoperoxidase by gel filtration on a Sephacryl S-200 column (1 × 30 cm) that was previously equilibrated with 0.05 *M* phosphate-buffered saline containing 0.1% bovine serum albumin (pH 7.6). The specific radioactivity of the ^{125}I-labeled EGF ws usually between 80 and 100 μCi.

Normal lung and lung-tumor crude membrane preparations were isolated in a manner similar to the method described by Leung (13). Lung tissues (average, 5–10 g) were homogenized in 0.3 *M* sucrose, and the homogenate was centrifuged at 11,000*g* for 15 min. The resultant supernatant was subjected to further centrifugation at 100,000*g* for 60 min. The resulting pellet was termed crude membrane preparation and was resuspended in Tris-HCl buffer (25 m*M* Tris, 10 m*M* $MgCl_2$, and 0.02% NaN_3 at pH 7.5) for protein determination, as estimated by the Bradford reagent (Bio-Rad Co., Richmond, CA).

For the binding assay, 300–500 μg of membrane protein were added in 100 μl of assay buffer (Tris-HCl buffer with 0.1% bovine serum albumin) along with 100 μl of ^{125}I-labeled EGF (100,000–130,000 cpm). Specific and nonspecific binding was determined by incubating ^{125}I-labeled EGF with receptor containing protein either in the presence or absence of unlabeled EGF. The unlabeled EGF, >1000-fold compared with ^{125}I-labeled EGF, at 100-μl volume, was added to give a final concentration of 3.3 μg/ml.

Competitive inhibition studies were performed with the various concentrations of unlabeled EGF. After incubation at room temperature for 16–20 hr, 1 ml of cold buffer was added for washing to each tube, and the tubes were immediately centrifuged at 4°C for 20 min at 1000*g*. The supernatant was decanted, and the bound ^{125}I-labeled EGF was counted in an automatic gamma counter (model 28023; Micromedic System, Horsham, PA). The results were calculated according to the method of Scatchard (14) and expressed in femtomoles per milligram of solubilized protein.

Each sample was tested in duplicate in all assays. Differences in EGF-R binding between the normal lung and lung-tumor tissues were analyzed by Student's *t* test, with $P < 0.05$ considered to be significant.

Results

The binding of ^{125}I-labeled EGF to normal dog lung crude membrane preparation increases linearly with the amount of receptor containing protein added (Fig. 1). Normally, each crude membrane preparation sample used in the binding assay consists of 300–500 μg of membrane protein per incubation. When we examine the EGF-R binding between tumor and normal dog lung tissues, the mean percentage of specific EGF-R binding from the dog lung tumors ($n = 8$) was almost 10 times higher than the EGF-R binding in normal lung tissue ($n = 13$): 31.38 ± 9.62% (mean ± SEM) versus 3.76 ± 0.91, $P < 0.001$. Specific EGF-R binding in individual lung-tumor tissue was compared with that in normal lung tissue from tumor-free dogs (Fig. 2). Specimens of lung tumors from five dogs had greater EGF-R binding than did the normal lung tissue; EGF-R binding in one tumor was not significantly different from that in normal tissue. Furthermore, when EGF-R binding in two lung tumors was compared with that in normal lung tissue obtained from a different lobe in the same dog, the percentage of specific EGF-R binding was also significantly higher in the tumor tissue (Fig. 3). Moreover, EGF-R binding in the "normal" tissue immediately adjacent to the tumor was not different from normal tissue in the other lobe in one dog (Fig. 3). In addition, the increase in EGF receptor number in dog lung tumors does not correlate with the increase in initial lung burden exposure to plutonium.

A displacement study revealed that purified EGF can inhibit the binding of ^{125}I-labeled EGF to normal and tumor dog lung tissues, and a representative result of three normal and tumorous dog lung samples is shown in Figure 4. There was only sufficient material for competitive inhibition multipoint analysis of bind-

Table I. Radionuclide Exposure and Tumor Classification for Dogs Exposed to Inhaled Plutonium

Identification[a]	Sex	Radionuclide	Initial lung burden (nCi)	Tumor type
A	F	$^{238}PuO_2$	22	Bronchioloalveolar carcinoma
B	F	$^{239}Pu(NO_3)_4$	72	Papillary adenocarcinoma
C	F	$^{238}PuO_2$	140	Papillary adenocarcinoma
D	M	$^{239}Pu(NO_3)_4$	54	Papillary adenocarcinoma
E	M	$^{238}PuO_2$	17	Papillary adenocarcinoma
F	M	Control	0	Papillary adenocarcinoma

[a] Letters correspond to those shown in Figures 1 and 2.

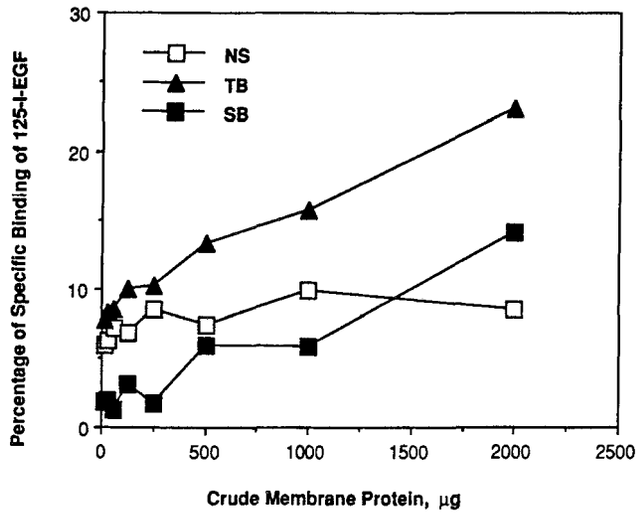

Figure 1. Effect of amount of crude membrane proteins on specific binding of ^{125}I-labeled EGF. NS, nonspecific; TB, total binding; SB, specific binding; $n = 3$.

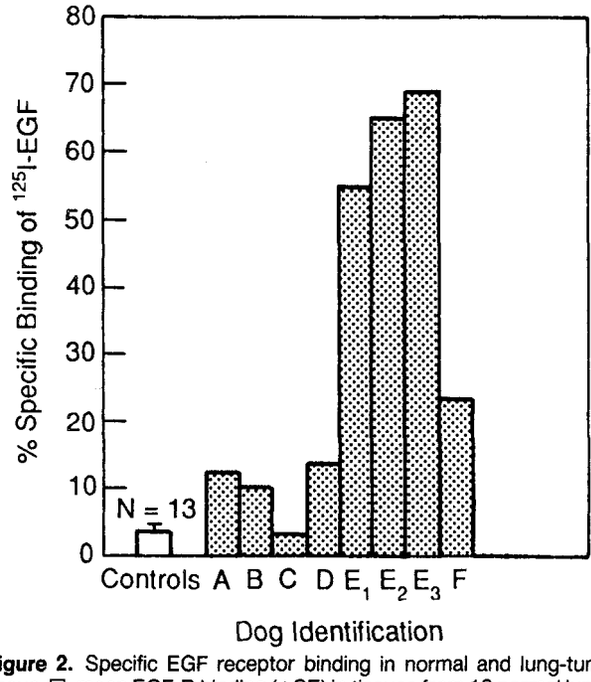

Figure 2. Specific EGF receptor binding in normal and lung-tumor tissue. □, mean EGF-R binding (±SE) in tissues from 13 normal lungs, ▨, EGF-R binding from lung-tumor tissue samples assayed in duplicate. Letters correspond to those in Table I; subscript numbers represent different tumor samples obtained from the same dog.

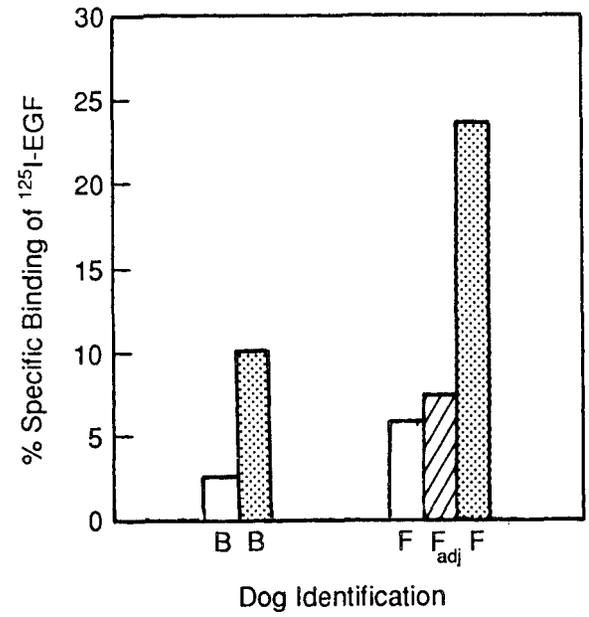

Figure 3. Comparison of specific EGF receptor binding in tumors assayed in duplicate (□, normal; ▨, lung tumor) obtained from the same dog. F_{adj} (▨), lung tissue immediately adjacent to tumor tissue.

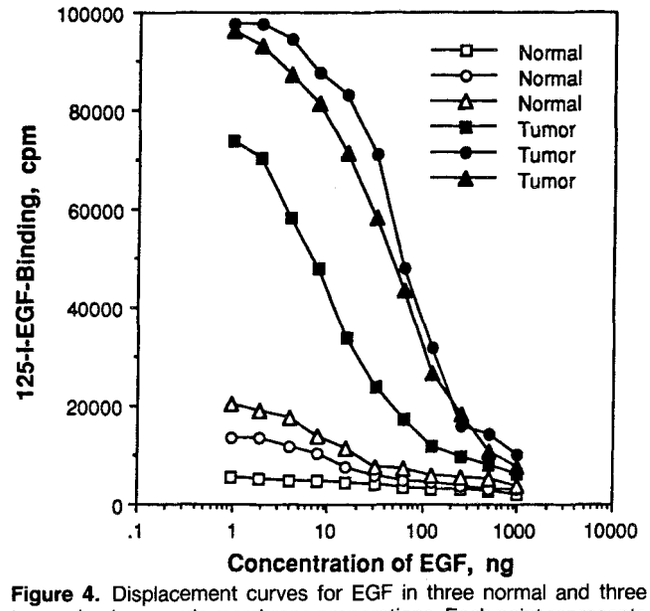

Figure 4. Displacement curves for EGF in three normal and three tumor dog lung crude membrane preparations. Each point represents the mean of duplicates.

ing on seven tumors and 12 normal lung membrane samples. Scatchard analyses of tumors and normal tissue are shown in Figure 5, and a summary of the binding characteristics is given in Table II. The Scatchard plot analysis was linear in all samples, suggesting a single class of receptor. The summary of the Scatchard plot analysis clearly indicated that the increase in EGF-R binding in the tumors was due to the increase in receptor sites without significant changes in receptor affinity. The crude membrane preparations from the lung tumors had approximately 10 times more EGF-R binding capacity than normal lung tissue.

Discussion

Lung cancer, a common type of cancer in humans, is generally classified into four major histologic types: squamous carcinoma, adenocarcinoma, large cell carcinoma, and small cell carcinoma (SCCL) (15, 16). All but the last type are generally referred to as non-small-cell carcinoma and have different clinical and biologic

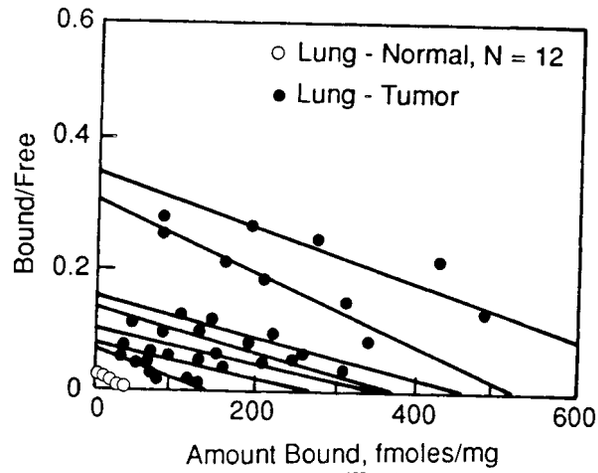

Figure 5. Scatchard plot analysis of ^{125}I-labeled epidermal growth factor binding to microsomal preparations of normal (○) and tumorous (●) lung tissue.

Table II. Binding Characteristics of Epidermal Growth Factor Receptors in Normal and Tumorous Dog Lung-Tissue Crude Membrane Preparations

	K_d (*M*)	Amount bound (fmol/mg)
Normal (n = 12)	$3.93 \pm 1.74 \times 10^{-8}$	53 ± 16
Tumor (n = 7)	$4.38 \pm 3.42 \times 10^{-8}$	619 ± 282

characteristics from small cell carcinomas. The latter is relatively common in human lung cancer (about 25%). Small cell carcinoma is extremely rare in all animal species, including the dog, therefore a good animal model for studying SCCL has not been established (17, 18). In addition, SCCL produce hormones and neuroendocrine hormones that have the characteristics of the endocrine cells of the amine precursor uptake decarboxylase (19). It is our experience that plutonium-induced lung tumors in beagles are non-SCCL (20).

Our observation that dog lung tumors have elevated EGF-R binding agrees well with data on human non-SCCL of the lung. Sherwin *et al.* (10) reported that EGF-R binding was higher than normal in five of the six non-SCCL cell lines examined, and that there was no detectable EGF-R binding in eight of eight SCCL cell lines examined. Gamou *et al.* (21) determined the presence of the EGF-R gene in non-SCCL (by Southern blot analysis), apparently in an intact and unarranged form. Using a monoclonal antibody to human EGF-R and an indirect immunoperoxidase staining techniques, Cerny *et al.* (7) reported that 80% of the 48 non-SCCL tissue samples examined stained positively, and that all 15 SCCL samples were negative with no staining. More specifically, abnormally high expression of EGF-R has been associated more often with squamous cell carcinoma than with other types of lung tumors (6), and EGF-R was not expressed in SCCL (6, 20). Hwang *et al.* (9) reported that in primary human lung tumors, squamous cell carcinomas and adenocarcinomas, EGF-R binding, and receptor autophosphorylation were elevated. Veale *et al.* (8) recently showed, by Scatchard analysis, that there was at least 5-fold more EGF-R in primary human non-SCCL tissue than in normal lung tissue.

At least nine epithelial cell types are present in the bronchial epithelium, and there are six cell types in the airway submucosal glands of humans and other species (22). Lung tumors in humans and other species are also heterogeneous, therefore it is not surprising that EGF-R binding varies in individual tumors. Immunocytochemical data are required to identify the specific cell type that has elevated levels of EGF-R as it was shown in human non-SCCL (7). We are in the process of determining which specific cell type possesses elevated EGF-R in radiation-induced dog lung tumors.

Our data represent the first demonstration of the involvement of EGF-R in non-SCCL in dogs, suggesting that EGF-R may play a role in the oncogenesis of plutonium-induced tumors as it does in lung tumors in humans. It is important to notice that the cause and effect relationship between an increase in EGF-R expression and non-SCCL lung tumors reported in human and in animals has not been established, and the mechanistic involvement of EGF-R expression remains to be determined. Our data also suggest that lung tumors in dogs may be used as animal models for studying growth factor/growth factor receptor in human non-SCCL.

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