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EGF receptor phosphorylation is affected by ionizing radiation

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Abstract

Eukaryotic cells respond to ionizing radiation with cell cycle arrest, activation of DNA repair mechanisms, and lethality. However, little is known about the molecular mechanisms that constitute these responses. Here we report that ionizing radiation enhances epidermal growth factor (EGF) receptor tyrosine phosphorylation in intact cells as well as in isolated membranes of A431 cells. Phosphoamino acid analysis revealed that ionizing radiation preferentially enhances tyrosine phosphorylation, while EGF enhances the phosphorylation of all three phosphoamino acids (serine, threonine and tyrosine) of the EGF receptor. In addition, radiation reduces the turnover rate of the EGF receptor, while EGF increases the rate of the receptor turnover and down-regulation. Moreover, the confined radiation-induced phosphorylation of tyrosine residues is inhibited by genistein, indicating that this phosphorylation of EGF receptor is due to protein tyrosine kinase activation. These studies provide novel insights into the capacity of radiation to modulate EGF receptor phosphorylation and function. The radiation-induced elevation in the EGF receptor tyrosine phosphorylation and the receptor's slower rate of turnover are discussed in terms of their possible role in cell growth and apoptosis modulation. © 1997 Elsevier Science B.V.

Keywords: Epidermal growth factor receptor; Tyrosine phosphorylation; Ionizing radiation

1. Introduction

The molecular basis of cell sensitivity or resistance to radiation is relatively unknown [1]. The contribution of signal transduction pathways to cell resistance or to cell sensitization to radiation is being extensively studied in several laboratories [2–8]. In addition to causing DNA damage, ionizing radiation upregulates early and late response genes and perturbs cell cycle [9,10]. Although the precise mechanism of radiation-induced cell arrest has not yet been elucidated [11], the involvement of growth factor signal transduction may be of fundamental importance in G1 arrest and apoptosis processes [12]. It has been shown recently in colorectal adenocarcinoma Difi cell, which, like A431 cells, express a high number of EGF receptors, that treatment with anti-EGF receptor antibodies induces both cell cycle arrest in G1 and apoptosis [13]. We have recently shown that anti-EGF

Abbreviations: EGF, epidermal growth factor; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; NCS, newborn calf serum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; PAS, protein A-Sepharose; TCA, trichloroacetic acid; EGTA, ethyleneglycol bis[β-aminoethyl]-N,N,N′,N′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; mAb, monoclonal antibody

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receptor antibodies enhance radiation-induced apoptosis [14].

EGF receptor has been detected in many kinds of human squamous cell carcinoma and normal squamous epithelium [15–18], and plays an important role in the proliferative processes of these cells [19]. This receptor is a transmembrane glycoprotein of 170 000 kDa [20–22]. Activation of the EGF receptor tyrosine kinase by EGF or TGFα results in a sequence of events involved in the mitogenic signal transduction pathways of cells [20]. Activation of the EGF receptor occurs in the following stages: binding of EGF to individual EGF receptors stimulates formation of non-covalent dimer (or oligomer) structures involving two (or more) receptors. The tyrosine kinase enzymatic activity of one receptor transphosphorylates residues on the opposite member of the pair. Once activated, the receptor initiates a series of signal transduction events via tyrosine phosphorylation of interacting proteins of the SH2 family [20,23–28]. Besides tyrosine phosphorylation, the in vivo EGF-activated receptors are phosphorylated on serine/threonine, by which receptor functions are attenuated. Various cellular protein kinases such as protein kinase C (PKC), mitogen-activated protein kinase, p34/cdk2 kinase, and casein kinase II are considered to be involved in this serine/threonine phosphorylation and receptor attenuation [29–32].

We have recently demonstrated that EGF receptor mediates resistance to radiation in A431 tumor cells [14]. However, it is still unclear how EGF receptor is affected by ionizing radiation. In experiments designed to explore this question, we measured receptor phosphorylation in intact A431 cells and in membrane preparations. We also studied the effects of radiation on EGF receptor turnover. Our present findings demonstrate that EGF receptor tyrosine phosphorylation is stimulated both when intact growing cells or isolated membranes are exposed to radiation, indicating that the machinery which transduces the effects of radiation resides in the cell membrane. Serine and threonine residues of the EGF receptor were not phosphorylated, and the half-life of the receptor was longer after radiation exposure. These results provide novel insights into the effects of radiation on the pattern of EGF receptor phosphorylation and function.

2. Materials and methods

2.1. Materials

EGF was from Collaborative Research (Waltham, MA), [^{35}S]methionine, [^{32}P]orthophosphate, and ^{125}I-labeled protein A were from Du Pont–New England Nuclear. Anti-phosphotyrosine antibody PY69 and genistein were purchased from ICN Biochemical (Cleveland, OH). Anti-phosphotyrosine antibody PY20 was purchased from UBI (Santa Cruz). Anti-EGF receptor monoclonal antibodies (mAbs) 528 and LA22 were kindly provided by Dr. J. Mendelsohn (Memorial Sloan Kettering Cancer Center, NY) and Dr. J.D. Sato (Alton Jones Center, Lake Placid, NY), respectively. Anti-EGF receptor carboxyl-terminal peptide anti-serum RK2 was kindly provided by Dr. J. Schlessinger (New York University Medical Center). All the other chemicals were from Sigma unless otherwise indicated.

2.2. Cell cultures and irradiation conditions

A431 cells were cultured in DME medium containing 10% fetal bovine serum ((FBS), Gibco Laboratories). For individual experiments, cells were seeded into 6-well plates in appropriate medium supplemented with 10% FBS and switched to 1% FBS the next day. The cells were then treated with either 5 nM EGF or with different doses of radiation, or with both. Proliferation was assayed by trypsinizing the cells and counting with a Coulter counter. Radiation was delivered to cells in a Gamma-cell 40 chamber containing two sources of 137Cs (Atomic Energy of Canada) at a dose rate of 100 cGy/min. Cells were exposed to single doses ranging from 200 cGy to 1000 cGy.

2.3. Western blot analysis of phosphotyrosine

A431 cells were cultured for the indicated times after exposure to radiation in the presence or absence of EGF, followed by removal of the culture medium and by rinsing the cells with cold PBS. Cells were lysed in a lysis buffer containing a mild detergent and protease and phosphatase inhibitors (50 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol, 1 nM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin,

10 μg/ml aprotinin, 2 mM sodium orthovanadate, 1.5 mM magnesium chloride, 1 mM EGTA) and incubated for 30 min at 4°C. The lysates were then mixed with concentrated SDS sample buffer to achieve final concentrations of 62.5 nM Tris–HCl, pH 6.8, 2% SDS, 0.5% 2-mercaptoethanol, 10% glycerol and boiled for 5 min. Samples were analyzed by 7% SDS–PAGE, western blotted, and nitrocellulose membranes were incubated either with anti-phosphotyrosine monoclonal antibody (mAb) PY69 or PY20 (at concentrations of 4 μg/ml), or with anti-EGF receptor peptide polyclonal antibody RK2, or anti-EGF receptor LA22 mAb (at a concentration of 1 μg/ml). Bound antibody was detected using ^{125}I-protein A [33]. Sample protein concentrations were equalized before loading onto the gels. Either whole lysate, or the immunoprecipitated (with 528 mAb) EGF receptor (see below) were transferred onto the nitrocellulose membrane, and incubated with anti-phosphotyrosine antibody (either PY69 or PY20).

2.4. Cell metabolic labeling

A431 cells were plated in 10% FBS DME medium for 2 days to reach approximately 70% confluence. For [^{35}S]methionine labeling, cells were switched to the labeling medium (90% methionine-free DME medium, 10% regular DME medium, 100 μCi of [^{35}S]methionine/ml and 1% dialyzed FBS) for 16 h. After removing the labeling medium, the cells were rinsed twice with fresh medium and cultures were chased in 1% dialyzed FBS DME medium for varying periods of time in the presence of EGF or in the absence of EGF after exposure to ionizing radiation. For [^{32}P]orthophosphate labeling, cells were grown for 16 h in phosphate-free DME medium with the addition of 300 μCi of [^{32}P]orthophosphate/ml, 4 mM glutamine, 1 mM sodium pyruvate, and 1% dialyzed FBS. Cells were exposed to radiation, or EGF was added during the final 30 min of labeling [33]. After incubation for the indicated times, cells were rinsed with cold PBS, incubated in Triton X-100 lysis buffer, and subjected to EGF receptor immunoprecipitation as described below.

2.5. Immunoprecipitation

A431 cells in Triton X-100 lysis buffer were incubated for 30 min in an orbital shaker at 4°C. To remove insoluble material, cell lysates were centrifuged at 14 000 rpm for 5 min at 4°C, and supernatants were pre-cleared by adding 50 μl of 20% pansorbin (Calbiochem) to each sample and incubating for 1 h at 4°C. Aliquots of supernatants containing equal amounts of protein were immunoprecipitated for 2 h at 4°C with anti-EGF receptor mAb 528/rabbit anti-mouse IgG (Accurate Chemical and Scientific Corp.)/protein A–Sepharose (Pharmacia LKB Biotechnology) conjugate [33].

2.6. In vitro phosphorylation

Membrane fractions were prepared and in vitro phosphorylation assay was carried out as previously described [33]. Briefly, cells were collected in the SAT buffer (0.25 M sucrose, 10 mM acetic acid, 10 mM triethanolamine, pH 7.4), and lysed in SEAT buffer (1 mM EDTA in SAT buffer), followed by centrifugation at 800 rpm for 5 min to pellet nuclei. The supernatant was centrifuged at 100 000 rpm for 15 min to pellet the membrane. The resulting pellet was resuspended in HEPES–Triton buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 0.2 mM EDTA, 10% glycerol) and centrifuged at 100 000 rpm for 15 min. The supernatant containing the solubilized membrane fraction was used. The reaction mixture for the in vitro phosphorylation assay (final volume: 30 μl) contained 20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 5 μg bovine serum albumin, 5 μM ATP, 10 μg membrane protein. The reaction was initiated by the addition of ^{32}P-ATP (in a total of 5 μM ATP), incubated for 10 min, and terminated by the addition of Laemmli's sample buffer.

2.7. Analysis of phosphoamino acids by two-dimensional electrophoresis

Two-dimensional phosphoamino acid analysis of EGF receptors labeled metabolically with [^{32}P]orthophosphate was performed as described previously [33]. Briefly, the polyacrylamide gel band containing EGF receptors was excised and homogenized. Labeled EGF receptors were precipitated with 15% trichloroacetic acid and partially hydrolyzed in 100 μl of 6 N HCl at 110°C for 1 h followed by three cycles of washing with deionized water and lyophilization. The recovery of total labeled material in

individual samples was 65–70%. The hydrolysate was subjected to two-dimensional thin-layer electrophoresis. The radioactivity associated with individual phosphoamino acids was measured by scrapping spots from the thin-layer plate and counting in a β-counter.

3. Results

3.1. Modulation of cell growth by radiation and by EGF

The growth of an epithelial tumor cell line, such as A431 cells, with elevated expression of EGF receptor, is inhibited by EGF [33,34]. As expected, and as shown in Fig. 1, addition of 5 nM EGF was antiproliferative for A431 cells. Exposure of these cells to radiation also resulted in inhibition of the proliferative rate as compared with exponentially growing untreated cells. The extent of radiation-induced growth inhibition was dose-dependent and was maximized at 2000 cGy. At the same time, the viability of the cells, as tested by Trypan blue, was not affected by doses of radiation in the range of 100–1000 cGy. When radiation and EGF were applied together, the antiproliferative effects were additive, indicating two independent effects. A simple additive response suggests a lack of interaction, in that true interactive effects would either yield synergy or antagonism.

3.2. Modulation of EGF receptor turnover by radiation and EGF

The observation of added effects of radiation and EGF upon A431 cell proliferation led us to search for alterations in EGF receptor function following radiation, and compare these changes to the effects of EGF. Pulse/chase experiments were carried out to determine the effects of the natural ligand, EGF, and radiation on EGF receptor turnover in A431 cells. Cells which had been prelabeled with ^{35}S methionine were cultured in fresh medium for varying time intervals after being supplemented with EGF or after exposure to ionizing radiation. SDS–PAGE patterns of immunoprecipitated EGF receptors demonstrated that the half-life of receptors in control A431 cells was approximately 13 h (Fig. 2). This fell markedly to a half-life of less than 6 h in the presence of EGF. On the other hand, for cells cultured after exposure to radiation, the half-life was increased to 17 h.

3.3. Modulation of EGF receptor tyrosine phosphorylation by radiation and EGF

A series of experiments was carried out to determine whether radiation of A431 cells results in

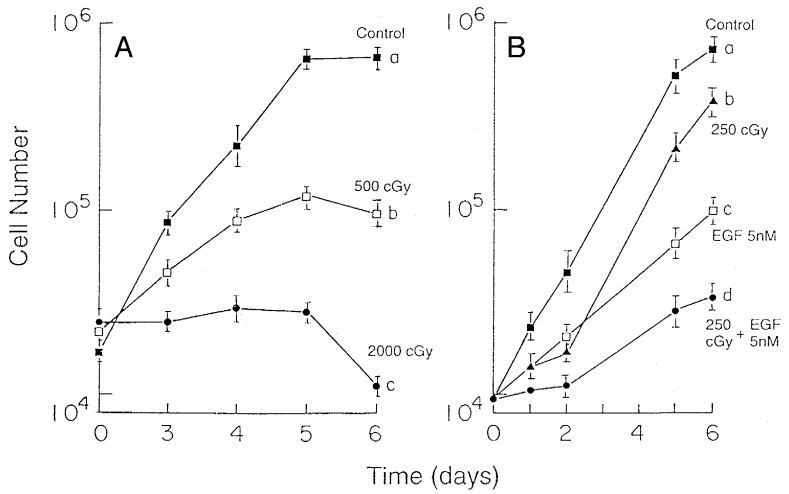

Fig. 1. Growth inhibition of A431 cells by radiation and by EGF. (A) Cells were seeded at low density in DME medium supplemented with 1% FBS. 24 h after seeding (day 0), cultures were untreated (a); treated with 500 cGy (b); treated with 2000 cGy (c). (B) Cells were seeded as described above. 24 h after seeding (day 0), cultures were untreated (a); treated with 250 cGy (b); supplemented with 5 nM EGF (c); both treated with 250 cGy and supplemented with 5 nM EGF (d). At the indicated times, cells were trypsinized and counted. The results are expressed as means of triplicates with standard error bars. The initial counts were 4×10^4 cells/well.

changes in phosphorylation of the EGF receptor. First, the effects of radiation were examined in intact cells either with whole cell lysates (Fig. 3) or after immunoprecipitation of the EGF receptor with anti-EGF receptor mAb 528 (Fig. 4). Then the effects of radiation exposure were studied on membrane preparations of A431 cells (Fig. 5) in order to investigate whether radiation can directly affect EGF receptor phosphorylation under cell free conditions.

As shown in Fig. 3, A431 cell cultures were exposed to different doses of radiation, lysates prepared, and equal amounts of total protein were subjected to SDS–PAGE analysis. Paired Western blots were immunodetected either with PY69 anti-phosphotyrosine antibody or with RK2 anti-EGF receptor antibody. Only anti-phosphotyrosine immunoblots show changes in the intensity of the phosphorylated tyrosines of the EGF receptor (PY69 immunoblots, Fig. 3A), while no changes are observed in the intensity of the receptor itself (RK2 immunoblots, Fig. 3B). Thus the amount of EGF receptor remains unchanged, while its tyrosine phosphorylation increases with elevated doses of radiation. As shown in Fig. 3A and 3C, EGF receptor tyrosine phosphorylation increased 3-fold after irradiation with a dose of 500 cGy, from 1 100 cpm *(lane 1, control)* to 3 500 cpm *(lane 3)*, and 4.5-fold after irradiation with a dose of 1 000 cGy, from 1 100 cpm (*lane 1, control*) to 4 950 cpm (*lane 4*). In comparison, EGF augmented tyrosine phosphorylation 5-fold to 5 700 cpm (*lane 5*), while EGF plus 500 cGy dose of radiation raised the receptor tyrosine phosphorylation 8-fold to 9 200 cpm (*lane 6*).

The kinetics of the effect of radiation on EGF receptor tyrosine phosphorylation is demonstrated in Fig. 3D. Cells were exposed to a constant dose of 500 cGy of radiation and then lysed at different time points for western blot analysis. As shown, EGF receptor tyrosine phosphorylation was stimulated 3-fold in 20 min after irradiation and remained elevated for several hours. We found that 3 h after irradiation the receptor tyrosine phosphorylation was still about 2-fold elevated as compared to control untreated samples (not shown).

These experiments were repeated with similar results when western blotting with anti-phosphotyrosine antibody (PY69) was performed after immunoprecipitation of the EGF receptor with anti-EGF receptor 528 mAb (Fig. 4). To further investigate the mechanism of EGF receptor tyrosine phosphorylation after radiation, we compared the responses to EGF and to radiation in the presence of genistein, a known specific inhibitor of protein tyrosine kinases [35–38]. Fig. 4B and 4C show that when A431 cells were cultured after radiation in the presence of different concentrations of the inhibitor, a genistein dose-dependent inhibition of EGF receptor tyrosine phosphorylation was observed. EGF-induced tyrosine phosphorylation of the receptor was also partially attenuated in the presence of genistein. These results provide additional evidence that radiation-induced tyrosine phosphorylation of EGF receptor is due to protein tyrosine kinase activation.

Finally, cell-free, in vitro, phosphorylation assay was carried out using the membrane fractions prepared from A431 cells (Fig. 5). As was shown previously with intact cells (Fig. 3 A and B), when the membrane fractions were exposed to radiation, en-

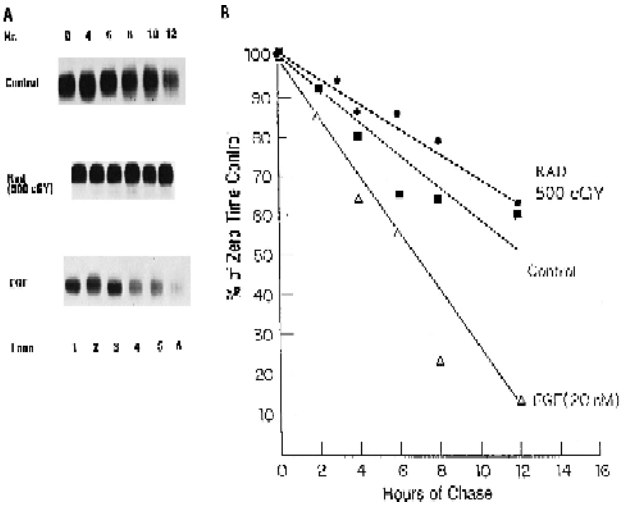

Fig. 2. Kinetics of EGF receptor turnover by radiation and by EGF. (A) A431 cells were labeled with ^{35}S methionine for 16 h. The labeling medium was removed, and cultures were chased in fresh DME medium containing 1% FBS for the indicated periods of time in the presence of 20 nM EGF or after irradiation with 500 cGy. EGF receptors were immunoprecipitated with 528 mAb and subjected to 7% SDS–PAGE and radio-autoradiography. Representative autoradiogram of one of three experiments. (B) The corresponding bands were excised and counted, and the plotted data were used to estimate the half-lives of labeled EGF receptors under the various conditions. Values (mean) represent data derived from duplicate points in three experiments. The S.E.M. of the values was 8%.

hancement of EGF receptor tyrosine phosphorylation was observed while the same amount of EGF receptor was present. This suggests that radiation directly affects membrane components to enhance the phosphorylation of EGF receptor tyrosine residues and thus its tyrosine kinase activity.

3.4. Phosphoamino acid two-dimensional analysis of the radiation-induced phosphorylation of the EGF receptor

When cells are incubated with EGF, phosphorylation of EGF receptors on tyrosine residues is accompanied by increased phosphorylation of serine and threonine residues [39]. Therefore it was important to investigate the effect of ionizing radiation upon the distribution of phosphorylation between these three phosphoamino acids. The next experiments were designed to measure radiation-induced changes in the specific amino acids phosphorylation of the EGF receptor (Fig. 6). A431 cells were equilibrium-labeled with [^{32}P]orthophosphate and then treated with EGF for 20 min at 37°C, or irradiated with a dose of 500 cGy 20 min prior to being lysed. The EGF receptors were immunoprecipitated from the lysates with 528 mAb and subjected to SDS–PAGE (Fig. 6A). EGF receptor bands were cut and hydrolyzed in HCl. The amino acids were separated by two-dimensional thin-layer electrophoresis, and ^{32}P incorporation into tyrosine, threonine, and serine was measured (Fig. 6B). Our results show that EGF stimulated a marked increase in phosphorylation of tyrosine, threonine, and serine, as expected. In contrast, ionizing radiation selectively stimulated tyrosine phosphorylation, with little increase in total phosphorylation (Table 1). Thus,

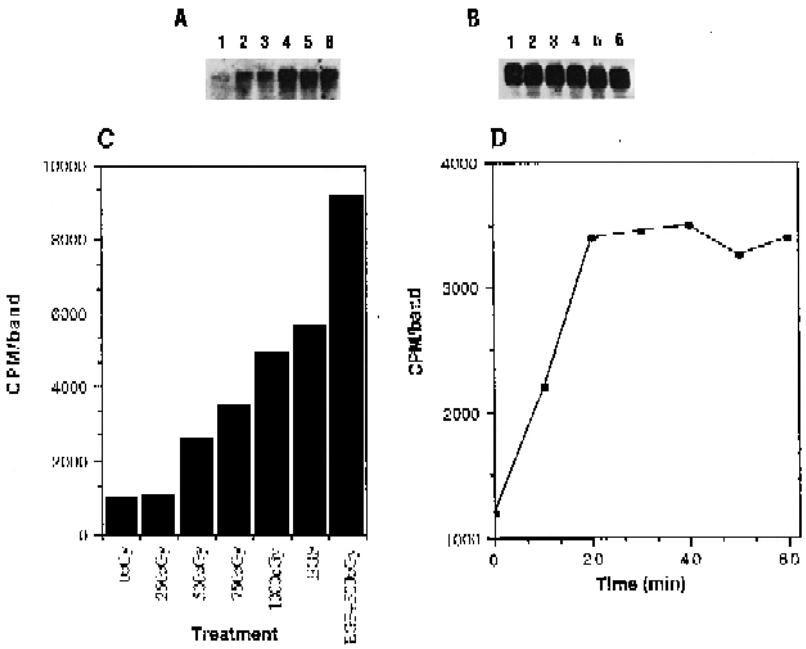

Fig. 3. Dose and time response of radiation-induced EGF receptor tyrosine phosphorylation. (A) and (B) A431 cell cultures (5×10^5 cells/35-mm well) were exposed to different doses of radiation. *Lane 1*, no treatment; *lanes 2, 3, 4*, irradiation with doses of 250, 500 and 1 000 cGy, respectively; *lane 5*, treatment with 20 nM EGF; *lane 6*, treatment with a dose of 500 cGy + treatment with 20 nM EGF. After 15 min at 37°C cell lysates were prepared in Triton X-100 lysis buffer, subjected to 7% SDS–PAGE, and immunoblotted either with PY69 (A) or with anti-EGF receptor antibody RK2 (B). After autoradiography and scanning by imaging densitometer the corresponding nitrocellulose bands were cut and counted in a β-scintillation counter. Both autoradiographies (representative autoradiogram of one of three experiments) of (A) and (B) are shown. (C) Presentation of EGF receptor tyrosine phosphorylation dose response to radiation demonstrated in (A). (D) Tyrosine-specific phosphorylation of EGF receptor in A431 cells was measured after exposure to 500 cGy dose of radiation and incubation at 37°C for the indicated time points. Values (mean) represent data derived from duplicate points in three experiments. The mean range of values in (C) was 5% and the S.E.M. values in (D) was 12%.

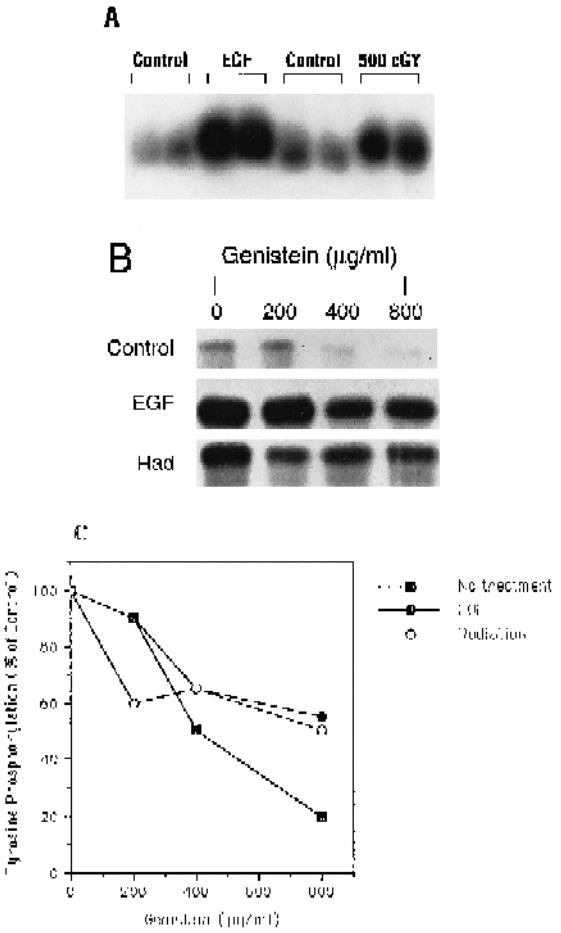

Fig. 4. Radiation-induced EGF receptor tyrosine phosphorylation analyzed after immunopreciptation with anti-EGF receptor antibody (A) and the effect of genistein on tyrosine phosphorylation (B and C). (A) A431 cells were untreated (control), treated with EGF (20 nM) or exposed to 500 cGy dose of radiation. After 15 min at 37°C, cell lysates were prepared in Triton X-100 lysis buffer, subjected to immunoprecipitation by the anti-EGF receptor 528 mAb, analyzed on 7% SDS–PAGE, and immunoblotted with anti-phosphotyrosine antibody PY69. Representative autoradiogram of one of three experiments. (B) A431 cells were untreated (control), treated with 20 nM EGF or exposed to 500 cGy dose of radiation as above. Increasing concentrations of genistein inhibitor were added at the time of irradiation or EGF exposure. Cell lysates were prepared in Triton X-100 lysis buffer containing genistein at the same indicated concentrations. The lysates were separated on 7% SDS–PAGE, and immunoblotted with anti-phosphotyrosine antibody PY69. Representative autoradiogram of one of three experiments. (C) After autoradiography the bands were scanned, and their relative intensities (in % of control (with no genistein) at time 0) are presented. Values (mean) are derived from triplicate determinations from one experiment representative of three similar studies.

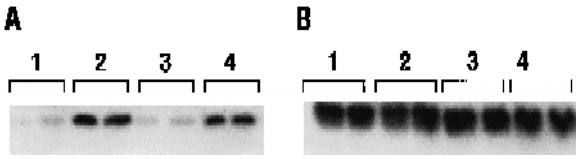

Fig. 5. The effect of radiation on the EGF receptor tyrosine phosphorylation in isolated membranes. Isolated membranes were treated with 20 nM EGF (*lane 2*) or exposed to radiation (500 cGy, *lane 4*). *Lane 1* is the control for EGF treatment, and *lane 3* is the control for radiation exposure. After 15 min at 37°C, membranes were solubilized in Triton X-100 lysis buffer, analyzed on 7% SDS–PAGE, and immunoblotted either with PY69 (A) or with anti-EGF receptor antibody RK2 (B). Representative autoradiogram of one of three experiments.

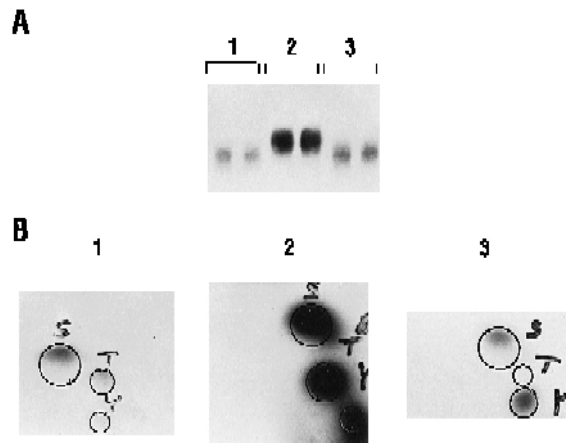

Fig. 6. Phosphoamino acid two-dimensional analysis of the EGF receptor. (A) A431 cells were labeled in phosphate-free medium with [^{32}P]orthophosphate at 37°C for 16 h. 30 min prior to the end of the labeling the following treatments were carried out: *lanes 1*, no treatment; *lanes 2*, 20 nM EGF; *lanes 3*, irradiation with a dose of 500 cGy. Subsequently, cells were lysed and the EGF receptor was isolated by immunoprecipitation and 7% SDS–PAGE. Representative autoradiogram of one of three experiments is shown. (B) The isolated receptors were eluted from the gel and subjected to partial acid hydrolysis. The ^{32}P-phosphoamino acids were resolved by two-dimensional thin layer electrophoresis, localized by autoradiography, and identified by ninhydrin staining of carrier molecules of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y). Representative autoradiogram of one of three experiments is shown. The amino acids were scraped and radioactivity was assayed by scintillation counting. The quantitative data are presented in Table 1. Values (mean) are derived from triplicate determinations from one experiment representative of three similar studies.

Table 1
Phosphoamino acid two-dimensional analysis

	Experiment	Counts/min (%)			
		Total	Tyr(P)	Thr (P)	Ser (P)
(1)	Control	6 320	1 270 (20%)	1 140 (18%)	3 920 (62%)
(2)	EGF (20 nM)	15 405	5 700 (37%)	3 851 (25%)	5 854 (38%)
(3)	Radiation (500 cGy)	6 600	3 040 (46%)	620 (9%)	2 980 (45%)

Phosphoamino acid analysis of immunoprecipitated EGF receptors was performed as described in Fig. 6. The proportion of each phosphoamino acid to the total of the three phosphoamino acids was determined for each group.

radiation-induced phosphorylation of the EGF receptor differs from that induced by the ligand, EGF.

4. Discussion

Single and repeated radiation exposures were shown to alter the expression of EGF receptor and estrogen receptor in MCF-7 cells [40]. It has also been shown that radiation resulted in a clear induction of EGF receptor expression in human keratinocyes [41], and that IGF-I receptor and/or EGF receptor function could be impaired by radiation exposure [42]. Other receptors may also be phosphorylated and activated upon radiation exposure. bFGF receptor tyrosine kinase has been reported to be involved in the protection of bovine aortic endothelial cells against the effects of ionizing radiation [2]. Two recent publications reported that Vasoactive Intestinal Peptide (VIP) receptors and adenylate cyclase activity in membrane isolated from pig jejunum were modified by irradiation [43], and that induction of inositol 1,4,5-trisphosphate receptor genes by ionizing radiation increased the level of Ins P3R type 1 protein [44]. So far, all these reports have not demonstrated any molecular data to provide mechanistic explanation regarding radiation effects on the respective receptors.

Our experiments provide new information regarding the molecular effects of ionizing radiation on the EGF receptor. We describe the notable increase in EGF receptor tyrosine phosphorylation and cell growth inhibition caused by ionizing radiation. This is not a peculiar phenomenon to A431 cells because it was observed in other cell lines including A549, derived from lung adenocarcinoma, and SKBR3, derived from mammary carcinoma (Goldkorn, unpublished).

The initial event after EGF binding is the autophosphorylation of its receptor on tyrosine residues. Studies of isolated membranes demonstrated the presence of at least five tyrosine phosphorylated residues in an EGF-activated receptor [45,46]. In contrast, studies of whole cells demonstrated a single tyrosine phosphorylation site of the EGF receptor isolated from EGF-treated cells [20,47]. In addition to tyrosine phosphorylation, serine/threonine phosphorylation is abundant and involved in the attenuation of the EGF-activated EGF receptor [29–32]. Thus, the low level of serine/threonine phosphorylation in radiation-activated receptor, may result in an inadequate diminution of receptor function.

The mechanism by which tyrosine phosphorylation of the EGF receptor is activated by radiation is not yet clear. EGF receptor autophosphorylation is thought to be a trans event, involving dimerization or oligomerization followed by tyrosine phosphorylation on the opposite receptor molecule [24,27,28,39]. Therefore, radiation could act by releasing EGF receptors from constraints which prevent dimerization in normal physiological conditions, such as intracellular inhibitors or structural elements attached to intact plasma membranes [48]. Another possibility is that radiation leads to separation of critically important tyrosine phosphatases from their association with the receptor kinase.

Receptor tyrosine phosphorylation induced by EGF could markedly be increased when exposed to radiation (Fig. 3). Therefore, the processes that contribute to radiation-induced tyrosine phosphorylation also have the capacity to enhance receptor activation mediated by its natural ligand. Moreover, our data provide strong evidence that radiation-induced EGF receptor phosphorylation results from protein tyrosine kinase activation because the presence of genistein, which is thought to be a competitor of ATP binding

to tyrosine kinases, can attenuate radiation-induced tyrosine phosphorylation. Radiation-induced EGF receptor tyrosine phosphorylation was reproduced using isolated membrane fractions from A431 cells. This suggests that ionizing radiation may activate the intrinsic tyrosine kinase of the EGF receptor and phosphorylate additional tyrosine residues that are only slightly phosphorylated under physiological conditions resulting from EGF stimulation. Alternatively, it is also possible that radiation activates an unidentified membrane tyrosine kinase(s) which is closely associated with the EGF receptor.

Because there was no stimulation of receptor serine/threonine phosphorylation by radiation, only minimal change in total receptor phosphorylation was observed (Fig. 6 and Table 1). The low level of serine/threonine phosphorylation may result in an insufficient attenuation of receptor function, such as lack of receptor internalization. Indeed, additional observations (Fig. 2) suggest that receptor turnover and down-regulation are far slower following cell exposure to radiation than to EGF treatment.

EGF receptor may not be the only membrane target for radiation. Our recent studies [14] have demonstrated an immediate decrease in cellular levels of ceramide in response to radiation. Under the same conditions, the cellular 1,2-diacylglycerol (DAG) levels decreased as well, being accompanied by the translocation of PKCα from the membrane to the cytoplasm. As also has been discussed [14], PKC down-modulates EGF receptor tyrosine kinase activity via EGF receptor Thr-664 phosphorylation [39]. Thus radiation-induced decrease in membrane PKC may further augment EGF receptor tyrosine kinase activity. The same may apply to radiation-induced ceramide modulation in A431 cells. We have shown [49] that ceramide down-modulates EGF receptor tyrosine kinase activity via the EGF receptor Thr-669 phosphorylation. Thus, radiation-induced decrease in A431 ceramide contents may further promote the EGF receptor tyrosine kinase activity. In sum, radiation-induced decrease in DAG cell contents followed by down-regulation of membranal PKC and the parallel decrease in ceramide levels may just amplify the net result of EGF receptor activation. It is interesting, however, that these membrane-associated molecular events cannot be generalized. It has been reported that exposure to radiation of bovine endothelial cells increased their cellular ceramide levels [8]. It has also been shown that radiation induced activation of PKC, as well as bFGF receptor in these endothelial cells [2].

The mechanisms by which radiation affects cell growth are as yet unknown. In an epithelial tumor cell line with elevated EGF receptors, EGF inhibits growth. Even though the capacity of EGF to inhibit the proliferation of cells expressing high levels of EGF receptor is well documented [13,33], the mechanism of this inhibition remains unexplained. There may be a quantitative relationship between EGF receptor kinase activity and growth response, and when an optimal kinase activation is exceeded, growth inhibition may result [33,34]. Cell cycle analysis suggested that both EGF and radiation cause G1 arrest in cycling A431 cells [11,33]. The EGF effect is consistent with growth inhibitory effects of nanomolar EGF on A431 cells [33], while the radiation effect is suggested to be additive with EGF-induced G1 arrest, consistent with the additive inhibitory effects of radiation and EGF treatment. These results pose an additional interesting question whether EGF receptor and its ligand could also contribute to programmed cell death.

We have recently [14] measured the initial apoptotic response of A431 cells to EGF, to radiation and to mAb (LA22) to EGF receptor. EGF alone slightly inhibited apoptosis, while LA22 slightly enhanced apoptosis in these cells. Moreover, radiation alone induced very low levels of apoptosis, but LA22 highly enhanced radiation-induced apoptosis by preventing the EGF receptor activation. We thus found that radiation-induced apoptosis could be enhanced by LA22. EGF, which activates the tyrosine kinase activity of EGF receptor, could delay mAb LA22-induced apoptosis. In sum, only combined treatment of mAbs to EGF receptor (which inhibit EGF receptor tyrosine kinase activity) together with radiation enhanced apoptosis. These results [14] demonstrated that in cancer cells such as A431, which overexpress the EGF receptor, radiation activates predominantly the EGF receptor to induce resistance to apoptosis, while anti-EGF receptor antibodies were shown to sensitize the cells to radiation by inducing apoptosis.

The present studies provide some mechanistic insights into the molecular effects of radiation on EGF receptor. As shown, the extent of tyrosine versus

serine/threonine phosphorylation in EGF receptor differs when cells are treated with EGF or with ionizing radiation, although both are growth-inhibitory stimuli. In contrast to EGF, ionizing radiation fails to modulate threonine/serine phosphorylation and to decrease the half-life of the receptor. As illustrated, radiation causes tyrosine phosphorylation without the accompanying effects of threonine/serine phosphorylation. It may thus create an aberrantly activated receptor which fails to be turned over in a regular manner. Such a receptor may antagonize apoptosis while still inhibiting cell growth.

The capacity of radiation to modify cell proliferation may partially reside in its ability to act as a direct biomodulator of the phosphorylation and function of the EGF receptor. This does not rule out a role for radiation as a biomodulator of other mechanisms involved in cellular growth. The finding that EGF receptor phosphorylation is affected by ionizing radiation and the distinction between the action of EGF and radiation on the EGF receptor pattern of phosphorylation may supply important clues toward the understanding of the mechanisms through which radiation interacts with regulatory signal transduction pathways.

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