Delayed Activation of Insulin-like Growth Factor-1 Receptor/Src/MAPK/Egr-1 Signaling Regulates Clusterin Expression, a Pro-survival Factor

Secretory clusterin protein (sCLU) is a general genotoxic stress-induced, pro-survival gene product implicated in aging, obesity, heart disease, and cancer. However, the regulatory signal transduction processes that control sCLU expression remain undefined. Here, we report that induction of sCLU is delayed, peaking 72 h after low doses of ionizing radiation, and is dependent on the up-regulation of insulin-like growth factor-1 as well as phosphorylation-dependent activation of its receptor (IGF-1 and IGF-1R, respectively). Activated IGF-1R then stimulates the downstream Src-Mek-Erk signal transduction cascade to ultimately transactivate the early growth response-1 (Egr-1) transcription factor, required for sCLU expression. Thus, ionizing radiation exposure causes stress-induced activation of IGF-1R-Src-Mek-Erk-Egr-1 signaling that regulates the sCLU pro-survival cascade pathway, important for radiation resistance in cancer therapy.

Ionizing radiation (IR) is commonly used for therapy against many cancers. Elucidation of refractory responses to clinically relevant doses of IR are under intense investigation. Understanding intracellular processes induced by IR that lead to radiation resistance, especially signal transduction pathways that precede gene expression, could reveal interventions that bring about a more favorable clinical outcome. Although it was once thought that the only important cellular responses to IR originated from DNA damage, it is clear that IR creates many different lesions in macromolecular targets within the cell that set in motion cascades of responses in both irradiated as well as in neighboring non-irradiated cells. For example, activation of epidermal growth factor and tumor necrosis factor-α receptors (i.e. EGFR and tumor necrosis factor receptor, respectively) after IR can stimulate intracellular signaling cascades that lead to gene expression that, in turn, mediates resistance to IR-induced cell death (1). IR may also stimulate the production of ligands (e.g. EGF, TGF-α, IGf-1, and tumor necrosis factor-α) that, in turn, activate downstream signaling (2–4). Both EGF and IGF-1 can activate the Src/MAPK signal transduction pathway (1). Because these signaling cascades can enhance survival of cancer cells, their stimulation can limit radiotherapy efficacy in various human malignancies that overexpress specific receptors. A better understanding of these signal transduction mechanisms is needed to improve radiotherapy.

Expression from the CLU gene encodes two protein forms, a non-glycosylated pro-death cytoplasmic/nuclear form (5) and another highly glycosylated secretory protein (sCLU) that provides cytoprotection after various genotoxic stresses, possibly due to its role as a molecular chaperone (6). sCLU and nuclearCLU proteins originate from separate mRNA species that can be targeted by siRNA approaches (5). sCLU expression is induced by a variety of cytotoxic agents (e.g. topotecan, taxol, and thapsigargin) (7); however, the signal transduction mechanisms regulating its induced expression remain a mystery.

sCLU has been implicated in many pathological states, including Alzheimer’s disease (8), atherosclerosis (9), rheumatoid arthritis (10), and cancer (11–15). High levels of endogenous sCLU in patients correlated with higher tumor grade and poor prognosis in prostate and breast cancers (15–17). Overexpression of exogenous sCLU resulted in resistance to paclitaxel (18), doxorubicin (19), cisplatin (20), and radiation therapy (21). In contrast, decreased sCLU expression by antisense or siRNA expression enhanced the chemosensitivities of various cell lines (22, 23), suggesting that sCLU expression was a prominent resistance factor in cancer cells.

We identified sCLU as an IR-induced protein/transcript (24). sCLU was induced by ≥2 cGy in various breast and colon cancer cells, with peak induction of sCLU noted 24–72 h after IR, as measured by promoter activity, transcript, and protein levels (7). Although specific transcription factors have been implicated in sCLU regulation after specific genotoxic stresses, including repression by c-Fos (25) and possible activation by c-Myc (19) and AP-1 (26), the signaling pathways involved and transcription factors that directly regulate the expression of this gene after IR have not been elucidated.

Using siRNA, we demonstrated a direct role for sCLU in cell survival after IR. Activation of the IGF-1/IGF-1R/Src/Mek/Erk
signaling cascade by clinically relevant doses of IR in MCF-7 breast cancer cells was required for sCLU induction. MAPK signaling was detected within minutes in cells after IR as reported (1); however, a dramatic re-activation of Src/MAPK signaling 24–72 h after IR was specifically required for sCLU promoter activation and elevated endogenous sCLU protein levels. A role for IGF-1/IGF-1r in sCLU induction after IR was elucidated. Because sCLU is a cytoprotective protein, these data define selective radio-resistant tumor cell phenotypes that overexpress and/or activate IGF-1/IR and offer specific mechanisms for therapeutic intervention.

EXPERIMENTAL PROCEDURES

Plasmids—Src CA, Src KD, dominant-negative (dn) Erk1, dnErk2, and Egr-1 plasmids were cloned into pcDNA3 (Invitrogen). Dr. Jeff Milbrandt generously provided the Egr-1 expression plasmid. A plasmid expressing dnMek1 were cloned into pcDNA3 (a gift from Dr. Richard G. Pestell, Georgetown University, Washington, D.C.), creating the CLU promoter-reporter, 4250-luc. pRSV-β-galactosidase (RSV-β-gal) was used as an internal control for transfection efficiency.

Cell Culture—Human MCF-7 breast and PC-3 prostate cancer cells were grown in RPMI 1640 medium with 5% fetal bovine serum at 37 °C in a humidified incubator with 5% CO2, 95% air. MCF-7 1403 cells that contain the human CLU promoter driving luciferase were described (7). Where applicable, MCF-7 cells were transiently transfected using Effectene (Qiagen; Valencia, CA) or Lipofectamine Plus (Invitrogen). All cell lines were mycoplasma-free.

Cell Treatments—PP1 was obtained from BioMol (Plymouth Meeting, PA), and U0126 was obtained from Cell Signaling Technology (Beverly, MA). AG1478, IGF-1, AG1024, and insulin-like growth factor-binding protein-3 (IGFBP3) were obtained from Calbiochem. EGF was obtained from Sigma.

The following experimental conditions were used for growth factor inhibitor treatments. Cells were serum-starved for 24 h and pretreated for 1 h with or without AG1478 or AG1024 in 0.01% Me2SO at indicated doses. Cells were mock- or IR-treated (5 Gy) as described (7) or treated with IGF-1 or EGF in serum-free medium. Medium was changed 1 h later to medium containing 1% serum plus inhibitors or ligands described above. After 24 h, medium was changed, and fresh 1% serum-containing medium was added every 24 h until harvest. For transfections, cells (1 × 107) were plated onto 35-mm dishes and transfected, and serum-starved. For Western blots, cells (5 × 105) were plated onto 10-cm dishes and serum-starved.

PP1 and U0126 treatments were performed on cells with similar density as noted above. Cells were pretreated for 1 h with PP1, U0126, or Me2SO and mock-irradiated or exposed to 5 Gy. In Fig. 5, A and B, medium containing μM PP1 was removed 24 h after IR. In all other experiments, continuous exposures of PP1 or U0126 were used, and new PP1 or U0126 in fresh media were added to cells every 24 h until harvest at 72 h or as indicated.

 Luciferase Reporter Assays—Luciferase reporter assays using co-transfection of 4250-luc with or without an RSV β-gal constitutive reporter were performed using the luciferase assay system (Promega; Madison, WI) as described (7). MCF-7 cells co-transfected with 4250-luc and Src CA, Src KD, dnMek1, or dnErk1/2 expression constructs were mock- or IR-treated, and CLU promoter-driven luciferase activities were assessed. MCF-7 cells were transfected 24 h before treatment with inhibitors and/or 5 Gy and harvested in 1× reporter lysis buffer at the indicated times. β-Galactosidase assays were used to control for transfection efficiency using 4250-luc. All experiments were normalized for protein using Bradford assays (Bio-Rad). Each dose/time point was completed in triplicate, and paired Student’s t tests were performed for statistical significance.

Western Blot Analyses and Co-immunoprecipitation—Whole cell extracts were prepared in radioimmunoprecipitation assay buffer, proteins were separated by 10% SDS-PAGE, and Western blot analyses were performed. Antibodies to human CLU-siRNA (S5, Ku70 (C-19), P-Mek1/2 (Ser421/822), Mek1 (C-18), P-Erk (E-4), Erk1 (K-23), P-Fak (Tyr925), and Egr-1 (588) were obtained from Santa Cruz (Santa Cruz, CA). IGF-1r-α, P-IGF-1r (Tyr1131), P-EGFR (Tyr1069), EGFR, and c-Src (Tyr416) antibodies were obtained from Cell Signaling Technology. α-Tubulin was obtained from Calbiochem. EGF was immunoprecipitated from 250 μg of total protein using 2 μg antibody at 4 °C overnight. Complexed lysates and antibodies were incubated with protein G-agarose beads at 4 °C for 1 h. Complexes were washed three times using SDS loading buffer, resuspended in SDS loading buffer, and boiled for 5 min, and proteins were separated by 12% SDS-PAGE. Relative protein band densities were determined from x-ray films of various exposures using NIH image, comparing treated to control signal densities and normalizing signals using an appropriate loading standard (either Ku70 or α-tubulin), where control values were set to 1.0.

Enzyme-linked Immunosorbent Assays—Enzyme-linked immunosorbent assays for IGF-1 were performed as directed (R&D Systems, Minneapolis, MN). MCF-7 cells (1 × 105) in 35-mm dishes were grown in medium without whole serum. Cells were mock- or IR-treated with 5 Gy 24 h later. At various times (0, 24, 48, and 72 h), medium (1 ml) was removed and stored at −80 °C. Samples (50 μl) were compared with an IGF-1 standard curve to determine concentrations. Results are the mean ± S.E. of three experiments, each performed in triplicate.

sRNA and Clongenic Survival—sRNA oligomers against the sCLU mRNA leader endoplasmic reticulum signal peptide (sCLU-siRNA) and a scrambled sequence (scr-siRNA) were synthesized by Dharmacon, Inc. (Lafayette, CO): sCLU-siRNA, 5′-GCC UGC AAA GAC UCC AGA AdTdT-T-3′ and 3′-dTdTCCG ACC UUU CUG AGC UCU-5′; scr-siRNA, 5′-GCC CGG CUG UUA GGA GGA GUA GGU AdTdT-T-3′ and 3′-dTdTCCG GGC AAA CAU CCG C-5′. sCLU-siRNA or scr-siRNA was trans- fected into MCF-7 cells followed by nuclear extract using Lipofectamine Plus (Invitrogen). Mock-transfected cells were used as a control. Two days after transfection, cells were trypsinized, plated onto 60-mm dishes (500 cells per dish) in triplicate, and mock- or IR-treated. Ten days later colonies containing >50 normal-appearing cells were counted.

Egr-1 oligomeric siRNA was made by Dharmacon, Inc. as a Custom SMARTpool containing four siRNA sequences against Egr-1 (Egr-1- siRNA). MCF-7 cells were transfected with 20 μM Egr-1-siRNA, 20 μM scr-siRNA, or mock-transfected using Oligofectamine (Invitrogen). After transfection (24 h), cells were exposed to 5 Gy or mock-irradiated. Cells were harvested in 1× reporter lysis buffer for luciferase assays or radioimmunoprecipitation assay buffer for Western analyses.

DNA Pull-down Assays—A biotinylated 1403-bp human CLU promoter plasmid was amplified from the 4250-luc plasmid using primers ordered from Integrated DNA Technologies (Corvalis, OR), 5′-GAT CCA TTC CCG ATT CCT-3′ and 5′-biotinylated-AGC CAA GCT TTC GGT GCC-3′. Nuclear extracts were harvested from Me2SO-, PP1-, or U0126-treated or mock-IR-exposed MCF-7 cells as described (27). Biotinylated CLU promoter (3 μg) was incubated with 10 μl of streptavidin beads (Oncogene Research Products; Boston, MA) for 1 h at room temperature. Complexes were washed (binding buffer) 50 μg Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 μg/μl poly(dI-dC)/poly(dI-dC), and 20% glycerol, and 10 μl of nuclear extract was added at room temperature for 20 min. Binding buffer (1 ml) was added, and complexes were incubated at 4 °C overnight. Complexes were washed twice 24 h later in binding buffer, and associated proteins were separated by 12% SDS-PAGE for Western analyses.

RESULTS

Activation of MAPK by IR—IR can activate both the EGFR and IGF-1R signaling cascades shortly after exposure. Because sCLU is expressed with delayed kinetics in all responding cancer cells (e.g. MCF-7) examined thus far after IR, with induction occurring 48–72 h after treatment (7, 28), we sought to elucidate whether MAPK signal transduction processes regulate such a delayed-induced gene product. As reported after high doses of IR (29), elevated levels of P-Src (Tyr416), P-Mek1/2 (Tyr292/294), and P-Erk1/2 (Tyr204) were noted, starting at 0.25 h and lasting for 2 h (Fig. 1, A and B). However, sCLU induction at these early time points was not observed. Interestingly, a dramatic re-activation of the Src/Mek/Erk1/2 pathway was noted 24–72 h after IR that correlated well with sCLU induction (Fig. 1, A and C). Thus, a delayed functional reactivation of the MAPK cascade correlated well with sCLU protein induction in irradiated MCF-7 cells.

EGFR Is Not an Upstream Activator of Clusterin—As noted above, IR can activate EGFR, resulting in the stimulation of Raf-Mek-Erk, MAPK signaling (4). We, therefore, examined a role for EGFR in CLU induction after IR using MCF-7 cells transiently transfected with a human CLU promoter-reporter
MAPK Involvement in IR-induced sCLU

MCF-7 cells transiently transfected with 4250-luc were treated with AG1024, a selective IGF-1R inhibitor. Exposure of cells to AG1024 significantly reduced sCLU promoter activity after IR compared with Me2SO controls (Fig. 3A). Furthermore, increasing doses of IGF-1 directly induced sCLU promoter activity, which was blocked by pretreatment with AG1024 (Fig. 3B). IR- and IGF-1-inducible sCLU promoter activities were also abrogated by IGFBP3, an endogenous inhibitor of IGF-1R (Fig. 3C). As expected, sCLU was not observed at earlier times (0–24 h) after 5 Gy or IGF-1 treatments (see Supplemental Fig. 1), but elevated sCLU protein levels were confirmed at 72 h post-treatment (Fig. 3D). Phosphorylation of IGF-1R dramatically increased 48 h after IR (Fig. 3E), and IGF-1R levels were elevated -40% in irradiated MCF-7 cells (Fig. 3F). Treatment of irradiated cells with AG1024 dramatically decreased IGF-1R levels (Fig. 3F), and AG1024 lowered basal levels of IGF-1R in non-irradiated, serum-starved cells. AG1024 also repressed basal levels of sCLU in PC-3 prostate cancer cells (see Supplemental Fig. 2), which are known to express high levels of IGF-1 and have elevated basal IGF-1R phosphorylation (31).

Elevated levels of phosphorylated IGF-1R in MCF-7 cells after IR suggested that IGF-1 production may be induced after IR (5 Gy). Media collected from serum-starved MCF-7 cells at various times post-IR showed elevated (~20%) levels of IGF-1, beginning 24 h after IR with sustained levels noted 72 h post-IR (Fig. 3G). Thus, MCF-7 cells have high basal IGF-1 levels, as reported (32), with increased levels of both IGF-1R and IGF-1 noted 24–72 h post-IR, consistent with enhanced phosphorylation of IGF-1R. These changes are consistent with a putative IR-induced IGF-1/IGF-1R autocrine feedback loop that, in turn, up-regulated sCLU levels.

c-Src Is an Upstream Activator of sCLU Induction after IR—
c-Src, but not other Src family members, can activate and be activated by IGF-1R (33, 34). PP1, a selective c-Src kinase inhibitor, abrogated CLU promoter induction after low doses of IR and partially blocked induction (by 50%) after higher doses of IR (Fig. 3A). As noted, sCLU was induced in MCF-7 cells by low doses of IR (0.1–5 Gy) (7), and PP1 (20 μM) blocked this induction (Fig. 3B).

We then determined the lowest dose of PP1 as a continuous 72-h treatment that could inhibit CLU promoter activity after 5 Gy. PP1 (5 μM) significantly decreased basal levels of the CLU promoter and sCLU protein and prevented IR-inducible CLU promoter activity (Fig. 3C) and endogenous sCLU protein levels (Fig. 3D). Phosphorylation of Fak, a known c-Src substrate (Tyr925), decreased with increasing PP1 doses (Fig. 3E). Thus, PP1 effectively blocked IR-inducible c-Src activity at doses that prevented sCLU protein induction.

To confirm a role for c-Src as an upstream regulator of sCLU induction after IR, we transiently overexpressed increasing amounts of constitutively active Src (Y529F) (Src CA) or kinase dead Src (K297R) (Src KD) cDNA constructs in MCF-7 1403 cells. MCF-7 1403 cells stably express 1403 bp of the CLU promoter linked to a luciferase reporter gene. Increasing levels of Src CA stimulated basal and IR-inducible CLU promoter activities (Fig. 4F, lanes 1–6). In contrast, increasing amounts of Src KD repressed IR-induced CLU promoter activity. These data, using PP1 and overexpression of Src CA or Src KD, illustrated a role for c-Src in sCLU induction in MCF-7 cells after IR.

Activation of the MAPK Cascade Is Required for sCLU Induction after IR—c-Src phosphorylates Raf, which in turn activates Mek-1 through phosphorylation (35). A selective Mek-1 kinase inhibitor, U0126 (1 μM), completely abrogated IR-inducible CLU promoter activity (Fig. 5A) and sCLU protein levels (Fig. 5B) in MCF-7 cells.

**FIG. 1.** Delayed activation of MAPK by IR. A, MCF-7 cells were mock- or IR (5 Gy)-treated, and whole cell extracts were harvested at various times (h) for Western analyses. Blots are representative of experiments performed three or more times. B and C, relative phospho-protein levels were determined by comparing treated over control levels using the appropriate total protein level as a loading standard. Relative sCLU protein levels were determined by comparing treated to mock-transfected cells, normalizing levels to α-tubulin, as in “Experimental Procedures.” In B, all data points are presented as the means ± S.E. Earlier times are presented in B, showing early activation of Src, Erk1/2, and Mek-1 but without sCLU induction. Each data point (means ± S.E.) was determined from three or more representative blots.
The Erk1/2 kinases are downstream substrates for Mek-1 (4). To demonstrate involvement of Erks in CLU gene induction, we overexpressed dnErk1 or dnErk2 in 1403 MCF-7 cells. Both dnErk1 and dnErk2 completely abrogated CLU promoter induction after 5 Gy (Fig. 5C).

Log-phase MCF-7 cells were co-transfected with 4250-luc and Src CA, Src KD, dnMek-1 K97A, or dnErk1 (Fig. 5D). In response to 5 Gy, CLU promoter activity was induced -4-fold in MCF-7 cells. Overexpression of Src CA resulted in significant elevation (4-fold) of CLU promoter basal levels compared with control. After IR, a further increase in CLU promoter activity was noted in Src CA-transfected MCF-7 cells. Overexpression of Src KD, dnMek-1, or dnErk1 abrogated IR induction of the CLU promoter. Western analyses confirmed overexpression of the corresponding proteins from the constructs (Fig. 5E). Collectively, these data demonstrated a role for MAPK signaling in sCLU induction in MCF-7 cells after IR.

Egr-1 Is Required for CLU Promoter Activation after IR—Several transcription factors are activated by the IGF-1R/MAPK-signaling cascade, but only a few are stimulated after IR (36). The CLU promoter contains three potential Egr-1 binding sites. Egr-1 is a known downstream target of MAPK (37) and can be activated by high doses of IR in lymphoid tumor cells (38). Overexpression of Egr-1 increased CLU promoter activity -2-fold over basal levels, and an -3-fold increase was noted in MCF-7 cells after 5 Gy (Fig. 6A). Western blot analyses confirmed expression of Egr-1 in these cells (Fig. 6B). These data suggested that Egr-1 was an important transcription factor that may mediate CLU induction after IR.

DNA pull-down assays were then performed to determine if Egr-1 directly associated with the 1403 CLU promoter and whether IR exposure enhanced Egr-1 DNA binding. Egr-1 DNA binding was minimal in control, mock-irradiated MCF-7 cells. An increase in Egr-1 binding was noted 4 h after IR, with more robust increases observed at 24 h and sustained binding of Egr-1 noted through 72 h post-IR. In contrast, IR-treated MCF-7 cells exposed to either PP1 (5 μM) or U0126 (5 μM) showed no increase in Egr-1 DNA binding to the CLU promoter DNA after IR. Nuclear extracts (10% input loaded) were separated by SDS-PAGE and probed for PCNA as a loading control (Fig. 6C).

We then examined whether IGF-1R or EGFR inhibition could block IR-inducible Egr-1 binding to the CLU promoter, placing the MAPK pathway in between. As expected, exposure of IR-treated MCF-7 cells with AG1478 (1 μM) did not affect Egr-1 DNA binding activity to the CLU promoter (Fig. 6D). In contrast and consistent with our observations of IGF-1R mediating sCLU induction after IR, Egr-1 DNA binding activity was blocked by co-addition of AG1024 (1 μM). Egr-1-specific binding to the CLU promoter was demonstrated by competition assays (Fig. 6E).

A role for Egr-1 in sCLU induction was confirmed using Egr-1-specific siRNA (siRNA-Egr-1). Exposure of mock-transfected or MCF-7 1403 cells transfected with scrambled siRNA (scr-siRNA) to IR resulted in an -6-fold induction of the 1403 CLU promoter (Fig. 6F). In contrast, CLU promoter activity was not significantly stimulated in Egr-1 siRNA (20 nM)-transfected MCF-7 1403 cells after 5 Gy. Efficacy of knockdown was confirmed by dramatic decreases in Egr-1 protein levels only.
after transfection with Egr-1 siRNA (Fig. 6G). Thus, decreased Egr-1 protein levels abrogated induction of CLU promoter activity at 72 h in MCF-7 1403 cells after IR.

siRNA to sCLU in MCF-7 Cells Enhanced IR Lethality—Cancer cells treated with a variety of chemotherapeutic agents induce sCLU, and its expression provides cytoprotection (19, 22).
To determine whether sCLU provides a similar cytoprotective role after IR, 20 μM siRNA oligomers specific to exon II of the sCLU mRNA were transiently transfected into MCF-7 cells. sCLU-siRNA transfected MCF-7 cells demonstrated a significant increase in clonogenic lethality with increasing IR doses compared with mock-transfected cells (Fig. 7A). In contrast, transfection with scrambled siRNA oligomers (scr-siRNA) did not alter the survival of irradiated MCF-7 cells (Fig. 7A) and only slightly decreased sCLU protein levels (Fig. 7B). sCLU protein levels were decreased 70% in MCF-7 cells using siRNA specific to sCLU (Fig. 7B), whereas only a minor change in nuclear CLU (nCLU) protein levels was observed; nuclear CLU lacks exon II (5). Thus, selective decreases in endogenous sCLU levels significantly increased the lethality of IR-treated MCF-7 cells.

**DISCUSSION**

We demonstrated that delayed induction of sCLU, a pro-survival protein, was dependent on the novel re-activation of IGF-1R/Src/Mek/Erk signaling 24–72 h after IR exposure. This signaling pathway culminated in the transactivation of Egr-1,
a known stress-inducible transcription factor (model, Fig. 6).

Considerable attention has been given to EGFR inhibitors for cancer therapy alone and in combination with IR. EGFR is overexpressed or constitutively activated in many tumors including colorectal, breast, pancreatic, and ovarian cancers (39). EGFR activation may contribute to radio-resistance in various tumors, including glioblastoma multiforme and breast cancer cells by activation of Erk1/2 (40, 41). As a result, EGFR-targeted therapies have been proposed, including the use of monoclonal antibodies and small molecule inhibitors that target the kinase domain (42). Interestingly, the selective EGFR inhibitor, AG1478, did not block the pro-survival signal transduction pathway leading to CLU promoter induction or regulate sCLU protein levels after IR (Fig. 2).

In contrast, IGF-1R has been far less studied as a target for refractory radiotherapy. IGF-1R activation resulted in mitogenic growth and cell survival (43), and treatment of cells with IGF-1 provided protection from doxorubicin- and taxol-induced apoptosis (44). AG1024, a selective inhibitor of IGF-1R, blocked sCLU induction after IR. Exposure to IR also activated IGF-1R (Fig. 3) and was required for delayed sCLU expression. These data are consistent with a previous report that AG1024 treatment of MCF-7 cells enhanced cell death after IR (45). The specific cytoprotective role of sCLU after IR (Fig. 7) strongly suggests that activation of IGF-1/IGF-1R signaling is an essential survival pathway that can be targeted for radiotherapy.

MCF-7 cells produce and secrete IGF-1 (32), and IGF-1R is often overexpressed in breast cancer (46). Peripheral lymph

**Fig. 5. Activation of MAPK is required for sCLU induction after IR.** A, MCF-7 cells were transiently transfected with 4250-luc and pretreated with increasing doses of U0126 1 h before mock or IR treatment. Medium containing fresh U0126 was added to cells every 24 h. Cells were harvested 72 h after IR for luciferase activities. Experiments were independently performed three times, with means ± S.D. graphed, and p values determined by paired Student’s t tests. RLU, relative luciferase units. B, MCF-7 cells were pretreated for 1 h with increasing doses of U0126 and mock- or IR (5 Gy)-treated. Medium containing fresh U0126 was added every 24 h. Cells were harvested at 72 h for Western analyses, and fold induction was determined as irradiated versus Me2SO-treated unirradiated control cells. C, MCF-7 1403 cells transiently transfected with dnErk1, dnErk2, or both were mock- or IR-treated. Cells were harvested at 72 h for CLU promoter-luciferase assays. Experiments were independently performed three times, with means ± S.D. graphed, and p values were determined by paired Student’s t tests. The asterisks indicate significant statistical difference between transfected irradiated versus mock-transfected IR-treated cells. D, MCF-7 cells were transiently co-transfected with 4250-luc in combination with vector only (VO), Src CA, Src KD, dnMek-1, or dnErk1. Later (24 h) cells were either mock- or IR-treated. Cells were harvested at 72 h for relative CLU promoter-luciferase activities. Experiments were independently performed three times, with means ± S.D. graphed, and p values were determined by paired Student’s t tests. Asterisks indicate a significant statistical difference between transfected versus vector-only-transfected cells. E, Western blots (WB) confirmed expression of c-Src, Mek-1, or Erk1 in MCF-7 cells treated in D. dnErk1 expression was confirmed using an antibody directed to its hemagglutinin (HA) tag.
node stromal cells produce and secrete EGF and IGF-1, which in turn can increase the growth and survival of breast cancer cells (47). Thus, EGF and IGF-1 secretion by lymph nodes may be a factor in the tumorigenesis of neighboring breast tissue, especially for cells that have up-regulated expression of EGFR or IGF-1R by a paracrine mechanism. Consistent with a previous report (43), we showed induction of IGF-1R after IR (Fig. 3D), an increase in IGF-1R phosphorylation (Fig. 3E), and a 20% increase in IGF-1 secretion 24–72 h post-IR (Fig. 3E).

Our data suggest a possible autocrine feedback loop induced by IR, where irradiated cells up-regulate IGF-1 and simultaneously increase IGF-1R synthesis (Fig. 3F). Induction of IGF-1 and its receptor provide a plausible mechanism for the delayed kinetics of endogenous sCLU protein levels in most cells after IR.

Our data suggest that a delayed induction of the Src-Mek-Erk cascade, culminating in transactivation of Egr-1, is required for IR activation of the CLU promoter and up-regulation of sCLU protein levels. As previously noted, IR caused an increase in IGF-1R phosphorylation (Fig. 3E), and a 20% increase in IGF-1 secretion 24–72 h post-IR (Fig. 3E).
MCF-7 cells were mock-transfected or transfected with siRNAs specific to scrambled sCLU protein knock-down in MCF-7 cells 48 h after transfection. Experiments were performed in duplicate, and means ± S.D. were graphed in A. p values were determined, and treatments were compared using paired Student’s t tests. B. Western blot confirming siRNA-specific sCLU protein knock-down in MCF-7 cells 48 h after transfection. MCF-7 cells were mock-transfected or transfected with siRNAs specific to scrambled (Scr-siRNA) or sCLU (sCLU-siRNA) mRNAs, and sCLU, nuclear cyclin (nCLU), and α-tubulin protein levels were monitored. Relative sCLU protein levels were determined by comparing treated to mock-transfected cells, normalizing levels to α-tubulin, as described under “Experimental Procedures.” Blots are representative of experiments repeated three times.

early activation of MAPK in MCF-7 cells, but that did not result in sCLU up-regulation. Instead, a novel re-activation of this signaling cascade days after exposure correlated with endogenous sCLU level induction. The physiological relevance of biphasic activation of MAPK after IR is unknown, and possible links between these pathways are being explored. Our data strongly suggest that delayed activation of MAPK is critical to sCLU induction. The importance of this delayed IR-stimulated pathway could be related to the fact that IGF-1R production in MCF-7 cells can cause increased resistance to herceptin (a monoclonal antibody to the Her2/neu receptor)-induced cell death (48). Our data strongly suggest a role for IGF-1R signaling in radio-resistance. Thus, tumor cells that survive an initial phase of radiotherapy may develop resistance to EGFPR inhibitors as a result of delayed MAPK induction stimulating IGF-1 and IGF-1R synthesis and leading to expression of sCLU, a downstream factor. Recent development of the rapidly aging, p44 knock-out mouse and their reported increase in IGF-1/IGF-1R signaling (49) support this theory, since sCLU overexpression during replicative senescence (50) and after loss of p53 function have been reported (7). Thus, the IGF-1R/Src/MAPK/Egr-1 pathway appears to be an important pro-survival system in many biologically stressful and pathological conditions. At least one end product of this pathway is sCLU.

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**FIG. 7.** siRNA knock-down of sCLU in MCF-7 cells increases IR lethality. A, MCF-7 cells were transfected with scrambled or sCLU siRNAs, which were treated with Scr-siRNA or sCLU-siRNA 48 h after transfection. B, Western blot confirming siRNA-specific sCLU protein knock-down in MCF-7 cells 48 h after transfection.
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