DELAYED ACTIVATION OF IGF-1R/Src/MAPK/Egr-1 SIGNALING REGULATES CLUSTERIN EXPRESSION, A PROSURVIVAL FACTOR

Tracy Criswell\textsuperscript{1,2}, Meghan Beman\textsuperscript{1}, Shinako Araki\textsuperscript{1}, Konstantin Leskov\textsuperscript{1}, Eva Cataldo\textsuperscript{1}, Lindsey D. Mayo\textsuperscript{1} and David A. Boothman\textsuperscript{1,2}

\textsuperscript{1}Department of Radiation Oncology, \textsuperscript{2}Program in Molecular and Cellular Basis of Disease, Laboratory of Molecular Stress Responses, 10900 Euclid Avenue, WRB-3, Case Comprehensive Cancer Center, Cleveland OH 44106-7285

Running Title: MAPK Involvement in IR-Induced sCLU

Address correspondence to: David A. Boothman, Department of Radiation Oncology, 10900 Euclid Avenue, WRB-3, Case Comprehensive Cancer Center, Cleveland OH 44106-7285. E-mail: david.boothman@case.edu or Lindsey D. Mayo, E-mail: lindsey.mayo@case.edu

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 IR, 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, IR 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.

Introduction

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 intra-cellular 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-\(\alpha\) receptors (i.e., EGFR and TNFR, 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-\(\alpha\), IGF-1, and TNF-\(\alpha\)) that, in turn, activate downstream signaling (2-4). Both EGF and IGF-1 can activate the Src/MAPK signal transduction pathway (1). Since these signaling cascades can enhance survival of cancer cells, their stimulation can limit radiotherapy efficacy in various human malignancies that over-express 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 nCLU 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). Over-expression 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 $\geq 2$ 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 this gene’s expression 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 CLU promoter activation, and elevated endogenous sCLU protein levels. A role for IGF-1/IGF-1R in sCLU induction after IR was elucidated. Since sCLU is a cytoprotective protein, these data define specific radio-resistant tumor cell phenotypes that over-express and/or activate IGF-1R, and offer specific mechanisms for therapeutic intervention.

**Experimental Methods**

**Plasmids**

Src CA, Src KD, dnErk-1, dnErk-2 and Egr-1 plasmids were cloned into pcDNA3 (Invitrogen, Carlsbad, CA). Dr. Jeff Milbrandt generously provided the Egr-1 expression plasmid. A plasmid expressing dnMek-1 was a kind gift from Dr. Jeff Holt, Vanderbilt University. A -4250 to +1 bp region of human CLU promoter was cloned into pA3luc (a gift from Dr. Richard G. Pestell, Georgetown University, Washington, DC), creating the CLU promoter-reporter, 4250-luc. pRSV-β-galactosidase (RSV βgal) was used as an internal control for reporter assays.

**Cell Culture**

Human MCF-7 breast and PC-3 prostate cancer cells were grown in RPMI 1640 medium with 5% FBS 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 obtained from Cell Signaling Technology (Beverly, MA). AG1478, IGF-1, AG1024, and IGFBP3 were obtained from Calbiochem (La Jolla, CA). EGF was obtained from Sigma (St. Louis, MO).

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% DMSO 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 added every 24 h until harvest. For transfections, cells (1x10⁵) were plated onto 35-mm dishes, transfected, and serum-starved. For western blots, cells (5x10⁵) 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 DMSO and mock-irradiated or exposed to 5 Gy. In Figs. 5A and 5B, medium containing 20 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, dnMek-1 or dnErk-1/2 expression constructs were mock- or IR-treated and CLU promoter-driven luciferase activities assessed. MCF-7 cells were transfected 24 h before treatment with inhibitors and/or 5 Gy and harvested in 1X reporter lysis buffer at 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 Labs; Hercules, CA). Each dose/time point was completed in triplicate and paired Student’s t-tests performed for statistical significance.

Western Blot Analyses and Co-Immunoprecipitations
Whole cell extracts were prepared in RIPA buffer, proteins separated by 10% SDS-PAGE, and western blot analyses were performed. Antibodies to human sCLU (B5), Ku70 (C-19), P-Mek1/2 (Ser218/222), Mek 1 (C-18), P-Erk (E-4), Erk 1 (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 (Tyr 1068), EGFR, and c-Src (Tyr416) antibodies were obtained from Cell Signaling Technology. α-Tubulin was obtained from Calbiochem. EGFR was immunoprecipitated from 250 µg 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 in RIPA buffer, resuspended in SDS loading buffer, boiled for 5 mins, and proteins separated by 12% SDS-PAGE. Relative phospho-protein or specific protein levels 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 (ELISAs)
ELISAs for IGF-1 were performed as directed (R&D Systems, Minneapolis, MN). MCF-7 cells (1x10^5) 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 to an IGF-1 standard curve to determine concentrations. Results are mean ±SE of three experiments each performed in triplicate.

siRNA and Clonogenic Survival
siRNA oligomers against the sCLU mRNA leader ER signal peptide (sCLU-siRNA) and a scrambled sequence (scr-siRNA) were synthesized by Dharmacon, Inc. (Lafayette, CO):
sCLU-siRNA
5’– GCG UGC AAA GAC UCC AGA AdTdT–3’
3’–dTdTCGC ACG UUU CUG AGG UCU U–5’
scr-siRNA
5’-GCG CGC UUU GUA GGA UUC GdTdT-3’
3’-dTdTCCGC GCG AAA CAU CCU AAG C-5’
sCLU-siRNA or scr-siRNA was transfected into MCF-7 cells (5 µg to 5x10^5 cells/60 mm dish) 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 4 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 1X reporter lysis buffer for luciferase assays or RIPA buffer for Western analyses.

DNA Pull-Down Assays
A biotinylated 1403 bp human CLU promoter was amplified from the 4250-luc plasmid using primers ordered from Integrated DNA Technologies (Coralville, IA):
5’ GAT CCA TTC CCG ATT CCT 3’
5’ /5Bio/ AGC CAA GCT TCC TGT GCC 3’.
Nuclear extracts were harvested from DMSO-, PP1- or U0126-treated, mock or IR-exposed MCF-7 cells as described (27). Biotinylated CLU promoter (3 µg) was incubated with 10 µl strepavidin beads (Oncogene Research Products; Boston, MA) for 1 h at room temperature (RT). Complexes were washed in binding buffer (50 mM Tris-HCL pH 7.5, 5 mM MgCl2, 2.5 mM
EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 µg/µl poly (dI-dC)· poly (dI-dC) and 20% glycerol) and 100 µg nuclear extract added at RT for 20 mins. 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 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. Since 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 regulated such a delayed-induced gene product. As reported after high doses of IR (29), elevated levels of P-Src (Tyr416), P-Mek (Ser218/222) and P-Erk-1/2 (Tyr204) were noted, starting at 0.25 h and lasting for 2 h (Figs. 1A,B). However, sCLU induction at these early times 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 (Figs. 1A,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 sCLU induction after IR using MCF-7 cells transiently transfected with a human sCLU promoter-reporter construct (i.e., 4250-luc, a 4250 bp region of the sCLU promoter controlling the firefly luciferase reporter). Increasing doses of AG1478, a selective EGFR inhibitor, did not affect IR-induction of the sCLU promoter in serum-starved MCF-7 cells, 72 h post-IR (Fig. 2A). Furthermore, increasing amounts of exogenous EGF had no effect on sCLU promoter activity or sCLU protein synthesis (S1), suggesting that EGFR did not play a role in sCLU induction. These results were confirmed by western analyses, wherein sCLU was not induced by EGF or the EGFR inhibitor, AG1478 (Fig. 2B, Lane 4, 5). Time-course analyses confirmed these results (S1). Consistently, AG1478 attenuated EGFR activation (Fig. 2C) by IR, as monitored by the autophosphorylation of Y1068 of EGFR, but did not alter IR induction of sCLU (Fig. 2B, lanes 3,7). Therefore, we concluded that EGFR did not play a role in sCLU induction after IR.

IGF-1R Inhibition Abrogates sCLU Induction

Stimulated IGF-1R is a potent activator of MAPK (30), however, involvement of IGF-1R after IR has not been explored. Serum-starved MCF-7 cells transiently transfected with 4250-luc were treated with AG1024, a selective IGF-1R inhibitor. Exposure of cells with AG1024 significantly reduced sCLU promoter activity after IR compared to DMSO 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 insulin-like growth factor binding protein-3 (IGFBP-3), 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 (S1), but elevated sCLU protein levels were confirmed at later times 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 (S2), 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. 4A). 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. 4B).

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 (Fig. 4C) and endogenous sCLU protein levels (Fig. 4D). Phosphorylation of Fak, a known c-Src substrate (Y925), decreased with increasing PP1 doses (Fig. 4E). 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 over-expressed increasing amounts of constitutively active Src (Y529F) (Src CA) or kinase dead Src (K297R) (Src KD) cDNA constructs in MCF-7 1403 cells. 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 over-expression 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, that 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.

The Erk-1/2 kinases are downstream substrates for Mek-1 (4). To demonstrate involvement of Erks in CLU gene induction, we over-expressed dominant-negative Erk-1 (dn Erk-1) or Erk-2 (dn Erk-2) in 1403 MCF-7 cells. Both dn Erk-1 and dn Erk-2 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, dominant-negative Mek-1 (dn Mek-1 K97A), or dn Erk-1 (Fig. 5D). In response to 5 Gy, CLU promoter activity was induced ~4-fold in MCF-7 cells. Over-expression of Src CA resulted in significant elevation (4-fold) of CLU promoter basal levels compared to control. After IR, a further increase in CLU promoter activity was noted in Src CA-transfected MCF-7 cells. Over-expression of Src KD, dn Mek-1 or dn Erk-1 abrogated IR-induction of the CLU promoter. Western analyses confirmed over-expression 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). Over-expression 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 coaddition 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, 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 to 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; nCLU 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. 6H).

Considerable attention has been given to EGFR inhibitors for cancer therapy alone, and in combination with IR. EGFR is over-expressed 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 Erk-1/2 (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 for targeted radiotherapy.

MCF-7 cells produce and secrete IGF-1 (32), and IGF-1R is often over-expressed in breast cancer (46). Peripheral lymph node stromal cells produce and secrete EGF and IGF-1, that 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 upregulated 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-1/2 pathway, 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 early activation of MAPK in MCF-7 cells, but this 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 EGFR inhibitors as a result of delayed MAPK induction stimulating IGF-1 and IGF-1R synthesis and leading to expression of sCLU, a pro-survival factor (Fig. 7). Small molecule inhibitors of IGF-1R could allow combinatorial therapies to overcome Herceptin resistance by manipulating the IGF-1R survival pathway, leading to a down-regulation of sCLU, as well as possibly other down-stream factors.

Our data also suggest that therapies specifically focused on down-regulating sCLU levels should augment various cancer therapies. sCLU provides cytoprotection against chemotherapeutic agents in many cancer cell types (19,22), and we show that sCLU provides cytoprotection for IR-exposed MCF-7 cells (Fig. 7). sCLU expression is elevated in many tumors types, including prostate, esophageal, colorectal, renal, and breast cancers (11,15-17). IGF-1R and IGF-1 production are also elevated in many of the same tumor types, especially prostate tumors. CLU and insulin-like growth factor binding proteins were suggested as targets for antisense therapy against prostate cancer (22), although a connection between IGF-1R signaling and sCLU induction was not examined. Our data strongly suggest that such a connection exists and could be exploited for improved therapy.

It is intriguing to speculate a possible role for sCLU in bystander effects, after radiation or chemotherapeutic therapies. An increased production and secretion of sCLU by tumor cells into the lymph or vasculature system may provide a survival effect for neighboring or metastatic cancer cells. Additionally, secretion of IGF-1 by normal stromal or tumor cells may provide a means for sCLU upregulation.

IGF-1R/Src/MAPK signaling leading to expression of sCLU, a pro-survival protein, may have implications beyond cancer biology and therapy. Increased sCLU expression was noted in Alzheimer’s patient tissues, after heart attacks, and during replicative senescence. Thus, the pro-survival IGF-1R/Src/MAPK/Egr-1 signaling pathway may be involved in these processes to regulate sCLU expression needed for debris clearing and survival. Recent development of the rapidly aging p44 knockout mice and their recent reported increase in IGF-1/IGF-1R signaling (49) support this theory, since sCLU over-expression 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.

Acknowledgements
This work was supported by DOE Grant, DE-FG02-99EQ62724 to D.A.B. and US Army DOD Breast Cancer Post- and Pre-doctoral Fellowships to K.S.L and T.C. (DAMD17-01-1-0196 and DAMD17-01-1-0194, respectively). We thank the
Radiation Resource Core of the Case Comprehensive Cancer Center (P30 CA43703).

References:


Figure Legends:

**Figure 1. Delayed activation of MAPK by IR.**

(A) MCF-7 cells were mock- or IR (5 Gy)-treated and whole cell extracts harvested at various times (h) for western analyses. Blots are representative of experiments performed three or more times.

(B & 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 mean ± standard error. Earlier times are presented in (C), showing early activation of Src, Erk-1/2, Mek-1, but without sCLU induction. Each data point (mean ± standard error) was determined from three or more representative blots.

**Figure 2. EGFR is not an upstream activator of sCLU.**

(A) MCF-7 cells were transiently transfected with 4250-luc and RSV β-gal and serum-starved for 24 h. Cells were then treated with 0, 1 and 3 µM of AG1478 alone (white bars) or with 5 Gy (gray bars), or EGF (black bars) as described in ‘Experimental Procedures’. Cells were harvested at 72 h for relative luciferase activities using RSV β-gal for loading. An asterisk denotes significant statistical difference between irradiated cells and cells treated with AG1478 alone. Experiments were performed independently three times, with means ±SD graphed, and p-values determined by paired Student’s t-tests.

(B) Serum-starved MCF-7 cells were treated with IR, AG1478, EGF or in combinations as indicated. Whole cell extracts were prepared 72 h, western blot analyses performed, and relative levels determined. Blots are representative of experiments performed three or more times.

(C) MCF-7 cells were treated as described in Fig. 3B. EGFR was immunoprecipitated from cellular extracts 48 h after treatment and prepared for western analyses. Blots are representative of experiments independently performed three or more times.

**Figure 3. Inhibition of IGF-1R abrogates sCLU induction after IR.**

(A) MCF-7 cells were transiently transfected with 4250-luc and RSV β-gal as in Fig. 3A. Cells were incubated 24 h later in serum-free medium for 24 h and then pretreated for 1 h with DMSO or increasing doses of AG1024 as in ‘Experimental Procedures’. Whole cell extracts were harvested 72 h later and luciferase activities monitored. A single asterisk denotes significant statistical difference between irradiated and mock-irradiated samples. Experiments were independently performed three times, with means ±SD graphed, and p-values determined by paired Student’s t-tests.

(B) MCF-7 cells were transiently transfected with 4250-luc and serum-starved as in Fig. 3A, then treated with increasing doses of IGF-1 (0, 10, 50 ng/ml) alone or with AG1024 as indicated. Cells were harvested at 72 h and CLU promoter activities assessed. Single asterisks denote a significant statistical difference between control and IGF-1-treated samples. Double asterisks denote a significant statistical difference between samples treated with AG1024 + IGF-1 (black bars), and samples treated with IGF-1 alone (white bars). Experiments were independently performed three times, with means ±SD graphed and p-values determined by paired Student’s t-tests.

(C) MCF-7 cells were transiently transfected with 4250-luc, serum starved as in Fig. 3A, then mock-irradiated, and exposed to IR or IGF-1 with or without indicated IGFBP3 doses. Cells were harvested 72 h later for CLU promoter activities. A single asterisk denotes significant statistical difference between IR- or IGF-1-treatments and mock-irradiated control. Double asterisk denotes significant statistical difference between IR-exposed and IR + IGFBP3. Triple asterisk denotes significant statistical difference between IGF-1 alone and IGF-1 + IGFBP3. Experiments were independently performed three times, with means ±SD graphed and p-values determined by paired Student’s t-tests.

(D) Serum-starved MCF-7 cells were treated as in Fig. 2B, except that AG1024 and IGF-1 were used at the indicated doses. Whole cell extracts were prepared 48 h later and western analyses performed for sCLU levels. Relative levels were determined as treated compared to mock-treated samples as described in ‘Experimental Procedures’. Blots are representative of three or more independent experiments.
MCF-7 cells were exposed to 5 Gy or mock-irradiated. Cells were harvested 48 h later and separated by 10% SDS-PAGE and blots were probed for either P-IGF-1R or total IGF-1R.

Serum-starved MCF-7 cells were treated as in Fig. 3D. Cells were harvested for western analyses 48 h after IR or IGF-1 treatment. Relative levels were determined as treated compared to mock-treated samples as described.

Serum-starved MCF-7 cells were mock-irradiated or exposed to IR and IGF-1 levels assessed against an IGF-1 standard curve at various times as indicated. Experiments were performed in triplicate. Asterisk denotes statistical differences in IGF-1 levels between IR- and mock-treated controls (t=0).

Figure 4. c-Src is an upstream activator of sCLU after IR.

(MCF-7 1403 cells were pretreated for 1 h with PP1 or vehicle alone (DMSO) and exposed to various IR doses. PP1 was removed 24 h after IR, cells were washed with PBS, and fresh medium added. Cells were harvested 48 h later for CLU promoter activities. Experiments were independently performed three times, with means ±SD graphed and p-values determined by paired Student’s t-tests.

MCF-7 cells were treated with PP1, with or without IR as in Fig. 4A. Whole cell lysates were harvested and analyzed by westerns. Ku70 was used for loading, and fold-induction determined as DMSO-irradiated or PP1-irradiated samples compared to DMSO-unirradiated control.

MCF-7 cells were transiently transfected with 4250-luc and pretreated with various doses of PP1 for 1 h prior to IR. Medium containing fresh PP1 was added to cells every 24 h and cells were harvested 72 h for CLU promoter-luciferase assays. Experiments were performed three times and SD determined by Student’s t-tests. Asterisk indicates a significant statistical difference between unirradiated PP1-treated versus DMSO-treated controls (white bars), or irradiated PP1-treated versus DMSO-treated cells (black bars).

MCF-7 cells were pretreated for 1 h with increasing doses of PP1 and then mock- or IR-exposed to IR. Medium containing fresh PP1 was added to cells every 24 h. Cells were harvested 72 h for western analyses. Fold-induction was determined as PP1-treated and irradiated samples compared to DMSO-treated unirradiated control.

MCF-7 cells were treated as in Fig 4D, and fold-induction determined as PP1-treated and irradiated samples compared to DMSO-treated control cells.

MCF-7 cells were transiently transfected with plasmids expressing Src CA (1-6) or Src KD (7-12) and mock- or IR-treated as in Fig. 4D. Cells in 1, 4, 7 and 10 were transfected with vector only DNA. Cells in 2, 5, 8 and 11 were transfected with 0.2 µg of indicated DNA and cells in 3, 6, 9 and 12 were transfected with 0.3 µg of indicated DNA. All transfections contained a total of 3 µg DNA. Samples were harvested at 72 h and CLU promoter activities assessed. Experiments were independently performed three times and p values determined by Student’s t-tests. Asterisks in bars 2 and 3 indicate a significant statistical difference between unirradiated Src CA transfected vs. unirradiated mock-transfected cells. Asterisks in bars 5 and 6 indicate a significant statistical difference in irradiated Src CA transfected vs. irradiated mock-transfected cells. The asterisk in bar 12 indicates a significant statistical difference in irradiated Src KD transfected vs. irradiated mock-transfected cells.

Figure 5. Activation of MAPK is required for sCLU induction after IR.

MCF-7 cells were transiently transfected with 4250-luc and pretreated with increasing doses of U0126 1 h prior to 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 ±SD graphed and p-values determined by paired Student’s t-tests.

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 determined as irradiated vs. DMSO-treated unirradiated control cells.

MCF-7 1403 cells transiently transfected with dn Erk-1, dn Erk-2, 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 ±SD graphed, and p-values determined by paired Student’s t-tests.
Asterisks indicate significant statistical difference between transfected irradiated vs. 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, dn Mek-1, or dn Erk-1. Later (24 h) cells were either mock- or IR-treated. Cells were harvested at 72 h for relative CLU promoter-luciferase activities calculated using RSV ß-gal. Experiments were independently performed three times, with means ±SD graphed, and p-values determined by paired Student’s t-tests. Asterisks indicate a significant statistical difference between transfected vs. VO transfected cells.

(E) Western blots confirmed expression of c-Src, Mek-1 or Erk-1 in MCF-7 cells treated in Fig. 5D. dnErk-1 expression was confirmed using an antibody directed to its HA-tag.

Figure 6. Egr-1 drives CLU promoter activity after IR

(A) MCF-7 cells were transiently co-transfected with 4250-luc, VO, or Egr-1 cDNA. Transfected cells were mock- or IR-treated and harvested 72 h later. Luciferase assays were used to monitor CLU promoter activities. Experiments were independently performed three times, with means ±SD graphed and p-values determined by paired Student’s t-tests.

(B) Western blot confirming Egr-1 expression in MCF-7 cells treated in Fig. 6A.

(C) MCF-7 cells were pretreated for 1 h with DMSO (1-6), PP1 (7-12) or U0126 (13-18) before mock- or IR-treatment (5 Gy). Nuclear extracts were harvested at various times for Egr-1 DNA binding and relative levels determined as irradiated vs. control at t= 0 h. PCNA was used as a loading control and experiments were independently performed at least three times.

(D) MCF-7 cells were pretreated with DMSO (1-2), AG1024 (3-4) or AG1478 (5-6) for 1 h, then mock- or IR-treated (5 Gy). Nuclear extracts were harvested at 72 h for Egr-1 DNA binding and relative levels determined as irradiated vs. untreated (UT) samples. PCNA was used as a loading control.

(E) Egr-1 binding to biotin-labeled 1403 CLU promoter using cells treated as described in Fig. 7C was decreased by increasing amounts (0.0, 0.1 or 5.0 µg) of non-biotin labeled 1403 CLU promoter.

(F) MCF-7 1403 cells were mock-transfected or transfected with siRNA oligomers specific to Egr-1 or scrambled sequence, then mock- or IR-treated. Cells were harvested 72 h later for CLU promoter-luciferase assays. Experiments were independently performed three times, with means ±SD graphed and p-values determined by paired Student’s t-tests.

(G) MCF-7 cells were mock-transfected or transfected with scrambled- or Egr-1-specific siRNAs as in ‘Experimental Procedures’, and 24h later mock- or IR-treated. Egr-1 steady state levels were determined by western blotting. Blots are representative of experiments performed three times and relative Egr-1 levels determined.

(H) Model depicting delayed IR activation of the IGF-1R and Src/Raf/Mek/Erk cascade, culminating in transactivation of Egr-1 and sCLU gene and protein induction.

Figure 7. siRNA knock-down of sCLU in MCF-7 cells increases IR lethality.

(A) MCF-7 cells were transfected with scrambled or siRNA oligomers specific to the endoplasmic reticulum (ER) leader peptide in exon II of CLU. Two days later, cells were mock- or IR-treated at indicated doses and then assessed for change in colony forming ability. Three experiments were performed in duplicate and means ±standard deviation (SD) graphed in ‘A’. p-Values were determined and treatments 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, nCLU and -tubulin levels monitored. Relative sCLU protein levels were determined by comparing treated to mock-transfected cells, normalizing levels to ß-tubulin, as in ‘Experimental Procedures’. Blots are representative of experiments repeated three times.
Figure-1
Figure 3
Figure 4
**Figure 5**

A. Graph showing RLU/Protein (10^3) for different U0126 doses (μM) with UT and 5 Gy conditions.

B. Table showing Fold Induction of sCLU and Actin proteins under UT and IR (5 Gy) conditions.

C. Graph showing RLU/Protein (X10^3) for UT and 5 Gy conditions with different Erk-1 and Erk-2 conditions.

D. Graph showing RLU/Protein (X10^3) for UT and 5 Gy conditions with different Src, CA, KD, Mek-1, and Erk-1 conditions.

E. Western Blot images showing WB: Src, Mek-1, and Ku70 for different VO, CA, KD, and Erk-1 conditions under IR (5 Gy) (-) and (+) conditions.

* p<0.05, * p<0.02
Figure 7

A

Surviving Fraction

Dose (Gy)

Mock

Scr-siRNA

sCLU-siRNA

* p<0.01

B

Mock-transfected

sCLU-siRNA

Scr-siRNA

Relative sCLU Levels:

1.0

0.3

0.8

nCLU

sCLU

α-tubulin
Supplemental Figure Legends:

Figure S1: sCLU is induced 24-72 h after IR or IGF-1 treatments, and is not blocked by pretreatment with AG1478.
MCF-7 cells (5x 10^5) were plated in 10 cm dishes. Normal growth medium was removed 24 h later and replaced with serum-free medium. Cells were pretreated for 1 h with or without AG1478 in 0.01% DMSO at the indicated doses. Cells were mock- or IR-treated (5 Gy) as described (Criswell et. al., 2003a), or treated with IGF-1 or EGF in serum-free medium. One hour thereafter, medium was changed to medium containing 1% serum plus inhibitor or ligands described above. After 24 h, medium was changed and fresh 1% serum-containing medium added (every 24 h) until harvest. Cells were harvested for western analyses at indicated time points (0, 4, 8, 24, 48, 72 h) after treatment. Relative levels were determined as treated samples compared to mock-treated control as in ‘Experimental Procedures’. Experiments were performed independently three times, with means ±SD graphed and p-values determined by the paired Student’s t-test.

Figure S2: AG1024 blocks sCLU induction after IR in PC-3 prostate cancer cells.
PC-3 cells (5 x 10^5) were plated in 10 cm dishes. Normal growth medium was removed 24 h later and replaced with serum-free medium. Cells were pretreated for 1 h with or without AG1024 in 0.01% DMSO at the indicated doses. Cells were mock- or IR-treated (5 Gy), or treated with IGF-1 in serum-free medium. One hour thereafter, medium was changed to medium containing 1% serum plus AG1024 or IGF-1. After 24 h, medium was changed and fresh 1% serum-containing medium added (every 24 h) until harvest. Western analyses were performed on extracts harvested 72 h after treatment. Relative levels were determined as treated samples compared to mock-treated control as described in ‘Experimental Procedures’. Blot represents experiments performed three or more times.
PC3 Cells

IR (5 Gy)   -   +   +   -   -   -   -
AG1024 (1.0 μM)  -   -   +   +   -   +   +
IGF-1 (10 ng/mL)  -   -   -   -   -   +   +

Relative sCLU Levels: 1.0  0.9  0.2  0.5  1.3  0.2
Lane: 1  2  3  4  5  6

sCLU
α-tubulin
Delayed activation of IGF-1R/Src/MAPK/Egr-1 signaling regulates clusterin expression, a pro-survival factor
Tracy L. Criswell, Meghan Beman, Shinako Araki, Konstantin Leskov, Eva Cataldo, Lindsey D. Mayo and David A. Boothman

J. Biol. Chem. published online February 2, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412569200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/02/18/M412569200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/02/02/jbc.M412569200.citation.full.html#ref-list-1