Enhanced Sensitivity of Human Oral Carcinomas to Induction of Apoptosis by Selenium Compounds: Involvement of Mitogen-activated Protein Kinase and Fas Pathways

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ABSTRACT

Prospective studies and recent intervention trials suggest that the risk of some cancers, including respiratory tract cancers, may be inversely related to selenium (SE) intake, and this is supported by strong experimental evidence with chemical-induced animal cancer models. How this cancer-protective effect is mediated is unclear, but interference with the balance of growth/apoptosis during tumor outgrowth is one plausible hypothesis. In general, there is a correlation between the effectiveness of SE compounds as chemopreventive agents in vivo and their ability to inhibit cell growth and induce apoptosis in vitro. This study has investigated the signal transduction pathways affected by SE compounds in biopsies of normal human oral mucosa cells and human oral squamous carcinoma cells (SCCs), using a primary culture system. Two SE compounds were tested: selenodiglutathione (SDG), the primary metabolite of selenite and the most commonly used cancer-protective SE compound in animal models, and the synthetic SE compound, 1,4-phenylenebis(methylene)selenocyanate (p-XSC), one of the most potent chemopreventive pharmacological SE compounds. Three novel findings are reported: (a) SCCs were found to be significantly more sensitive to induction of apoptosis by SDG than normal human oral mucosa cells, though the differences were marginal with p-XSC; (b) both SE compounds induced the expression of Fas ligand (Fas-L) in oral cells to a degree that correlated with the extent of apoptosis induction; and (c) both SDG and p-XSC induced the stress pathway kinases, Jun NH2-terminal kinase (JNK) and p38 kinase, at concentrations causing apoptosis; p-XSC, and to a lesser extent SDG, also activated extracellular regulated kinases 1&2 (ERKs 1&2) and protein kinase B or Akt. To test their functional involvement, the effect of inhibiting each of these pathways on induction of apoptosis by SDG and p-XSC was determined in SCCs. Inhibiting the ERKs 1&2 or Akt pathways with specific chemical inhibitors (PD98059 or LY294002, respectively) did not affect the extent of apoptosis induced by SDG or p-XSC (with the exception of LY294002, which actually enhanced the level of induction of apoptosis by SDG). The JNK pathway appeared to be most important for induction of Fas-L and apoptosis because concentrations of SB202190 that inhibited activation of both the JNK and p38 kinase (but not ERKs 1&2) in SCC reduced the extent of induction of Fas-L and apoptosis by SDG and p-XSC, whereas lower concentrations that inhibited activation only of p38 kinase did not. This was confirmed by the fact that exogenous expression of a dominant negative deletion mutant of c-Jun (TAM67) reduced the induction of both apoptosis and Fas-L by SDG.

INTRODUCTION

There is currently much interest in the potential cancer-protective effects of SE. Human epidemiological evidence indicates a statistically significant inverse relationship between SE intake (which is primarily in the form of selenomethionine) and risk of cancer overall, particularly in men (1, 2). In terms of individual cancer types, the evidence from the largest studies is strongest for lung, esophageal, prostate, and gastric cancers, but there is little evidence of any link with breast or colon cancer, although the length of follow-up in most of these studies was quite short. Three intervention trials also suggest that SE supplementation in the form of selenized yeast, either alone or with other antioxidant vitamins, reduces the risk of cancer (1). The most recent randomized placebo-controlled intervention trial with men with a prior history of skin basal or squamous cell carcinoma found no evidence for a reduction in risk of recurrence of skin cancer with 200 µg/d SE, but total cancer rates in the treatment arm were reduced significantly (by 37%), as were the rates of lung, colorectal, and prostate cancers (3, 4). The strongest treatment effect was observed in subjects with the lowest plasma SE levels before supplementation.

This evidence for a cancer-protective effect of dietary SE in humans is supported by very convincing animal evidence indicating that a high dietary level of SE, usually in the form of sodium selenite, substantially reduces the incidence of a wide variety of animal cancers under conditions where animal growth and health are not affected (1, 5, 6). Most studies have observed the maximum cancer-protective effect at nontoxic levels considerably higher than normal nutritional levels, but there is also more limited and less consistent evidence that sub-nutritional SE deprivation increases cancer risk. SDG, the primary metabolite of selenite, is potent anticarcinogenic, but selenomethionine, the most abundant form of SE in natural foods, has generally been found to be less effective than selenite (reviewed in Ref. 1). However, plant foods also contain other SE derivatives, such as Se-methylselenocysteine, which is an effective cancer-protective agent in animal models (5). There is therefore considerable interest in developing novel synthetic SE compounds that may have greater chemopreventive properties than naturally occurring forms. In animal models, the most effective chemopreventive agents seem to be those that are metabolized directly to monomethylated SE metabolites (1, 5, 7), including aliphatic or benzyl selenocyanates, such as p-XSC (8–10), used in this study. In particular, SE derivatives like p-XSC are effective in animal carcinogenesis models in which selenite is not so effective, e.g., 4-(methylthiosemicarbazido)-1-(3-pyridyl)-1-butanone-induced lung cancers (10).

Thus, overall, the available evidence indicates a definite cancer protective effect of SE in animal models and probably also in humans. However, as noted above, the human intervention trials have used a complex SE source (selenized yeast) rather than specific SE compounds, and so it cannot be assumed that the mechanisms responsible for the cancer-protective effect in animals is necessarily relevant to the human studies (reviewed in Refs. 1, 2, 11, and 12). Arguably, the animal studies with selenite or synthetic SE derivatives may be more

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4 The abbreviations used are: SE, selenium; ERK, extracellular regulated kinase; NOMC, normal oral mucosa cell; SDG, selenodiglutathione; SCC, oral squamous carcinoma cell; P-1,3 kinase, phosphatidylinositol-3-phosphate kinase; p-XSC, 1,4-phenylene-

nebithylene)selenocyanate; GPX, glutathione peroxidase; JNK, Jun NH2-terminal kinase; EGF, epidermal growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; GSH, glutathione; MAPKAPK, mitogen activated protein kinase activated protein kinase; FADD, Fas-associated death domain; Fas-L, Fas ligand.
relevant to devising potential future chemoprevention strategies in high-risk humans than to the putative cancer protective effect of SE from natural human diets.

Several possible mechanisms have been proposed to explain the cancer-protective effects of SE compounds. They can modulate immune responses (reviewed in Ref. 1), but the detailed mechanisms involved require to be elucidated, and it remains to be established that this effect could generate a sufficient antitumor response at the levels of SE that are cancer-protective in vivo. A second hypothesis proposed that the chemopreventive effect of SE might be mediated by selenoproteins, such as the GPXs, eliminating tumor-promoting reactive oxygen species; however, this now seems less plausible because the activities of known selenoproteins seem to be saturated at a much lower dietary SE level than that required for the maximum chemopreventive effect of SE (13, 14). There is additional evidence that at least the cystosolic GPX1 is not involved because GPX1-null mice do not show any abnormal histopathologies up to 15 months of age (15, 16), although they are more sensitive to exogenous oxidative stress (17), and GPX1-transgenic mice are actually more sensitive to 7,12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate-induced skin cancer, rather than the reverse, hypothetically, because the generation of tumor-promoting, lipoxxygenase-derived peroxides is increased (18). However, thioredoxin reductase remains a possible candidate because it has been shown to be inhibited by protracted high SE levels, possibly attributable to diselenide bond formation at the selenocysteine in the active site (19). Additionally, new selenoproteins have been discovered recently (reviewed in Ref. 6), but it is not yet known what their functions are or the SE levels at which their activities are saturated. A third hypothesis is that SE compounds act as antipromotion agents, possibly by inducing apoptosis in initiated premalignant cells. This is suggested by animal evidence that selenium and p-XSC are effective if given after carcinogen in the early phase of tumor progression, although they may also reduce DNA damage by carcinogens recently reviewed in Refs. 1 and 5). It is also consistent with the fact that, in general, the relative efficacies of SE derivatives as chemopreventive agents in vivo parallel their growth inhibitory effects in vitro and their ability to induce apoptosis (20–24); moreover, this could operate in premalignant cells because it does not necessarily require a functional p53 pathway (10, 21, 25). Recent work showing that both inorganic and organic forms of SE induce gadd34, gadd45, and gadd153 (25, 26), and inhibit cdk2 activity (26) suggests that interference with cell cycle checkpoint controls is associated with growth arrest/apoptosis induced by SE compounds.

The work reported in this paper, therefore, tested directly whether SE compounds with the most pronounced cancer protective properties affect any of the signal transduction pathways that control cell growth, survival, and apoptosis. In view of the fact that lung and esophageal cancers are two of the cancer types in which the epidemiological evidence for a cancer-protective effect of SE is strongest, we have focused on human oral cancers because they share similar risk factors to lung and other head and neck cancers. To obtain data as relevant as possible to human SCCs, we have used a panel of primary cultures of biopsies of oral squamous cancers and NOMC, which we have characterized previously (27, 28). We have investigated primarily two of the most potent cancer-protective SE compounds in animal studies (the primary metabolite of selenite, SE), which we have characterized previously (27, 28). All cells were maintained on irradiated 3T3 feeders, either in FAD+ medium (1:3 Ham’s F-12/DMEM with 10% FCS and insulin, EGFr, transferrin, cholera toxin, hydrocortisone, and adenosine) in the case of normal cells or 10H medium (DMEM plus 10% FCS without added growth factors, except hydrocortisone) in the case of carcinomas (27, 28). Normal cultures were used within the first two to three passages from frozen stocks, before their growth rate deteriorated significantly. Oral cultures were used for experimentation while rapidly growing at not >70% confluence, after which the irradiated 3T3 feeders were carefully removed by treatment with PBS/0.02% EDTA, and the cultures were refed with normal medium. 3T3 cells were maintained in 10C medium (DMEM plus 10% donor calf serum). HeLa cells were maintained in defined DMEM (Beantson formulation; Life Technologies, Inc., Inchinnan, United Kingdom) supplemented with 10% FCS and 1 mM glutamine. Cells were maintained in culture for a maximum of 6–8 weeks before being replaced with cells from frozen stocks. All media used contained Bufferral (Sigma Chemical Co., Gillingham, United Kingdom) to maintain the pH at 7.0.

Chemicals. SDG was prepared as described previously (29), p-XSC was purified to 99.9% homogeneity as described by El-Bayoumy et al. (30). SB202190, SB203580, PD98059, and LY294002 were obtained from Calbiochem, Nottingham, United Kingdom.

**DNA Synthesis/Proliferation Assay.** 10 6 irradiated 3T3 cells were plated in 200 µl of 10C medium per microtiter well. After 4d, the medium was removed, and oral epithelial cells were added (10 4 in the case of normal cells or 5 10 4 carcinoma cells) in 100 µl of 10H medium. After 48 h, the medium was replaced with medium containing SDG or p-XSC and incubated overnight. Each well was then given 0.5 µCi of tritiated thymidine for 6 h, the medium was removed, and the cells were trypsinized and transferred onto a filter paper mat (printed filtermat A; Pharmacia) using a microtiter plate harvester (Skatron Combi Harvester, model 11900; LKB, Skatron, Norway). After adding scintillator, the mat was scanned and counted using a plate counter (model 1205 Betaplate; Pharmacia). Four replicate wells were used for each condition.

Control experiments showed that the irradiated feeders supported growth of the oral cells for the duration of the experiment but contributed an insignificant background thymidine incorporation (<5% of that of the oral cells). The radioactivity incorporated into DNA was calculated as a percentage of untreated cultures.

**Apoptosis Assays.** Apoptosis in the oral cells was measured by TUNEL staining using the Apoptag kit (Intergen, Purchase, NY), according to the manufacturer’s protocol. The irradiated 3T3 feeders were carefully removed from the cultures before experimentation by washing the cultures vigorously three times with PBS/0.02% EDTA. Assays were performed with two to three replicate plates of cells, and ≤10 randomly selected fields per plate were counted for TUNEL-positive cells. Assessment of apoptosis among the HeLa cells transiently cotransfected with a GFP-encoding vector was performed by measuring the sub-G0/G1 DNA content of the GFP-positive population. Cells were harvested after treatment, washed in cold PBS, fixed in 1% paraformaldehyde for 1 h on ice, and permeabilized with 70% ethanol for 1 h on ice. Cells were then resuspended in 1 ml of PBS to which 0.5 ml of Phosphate-citric acid buffer was added and incubated at room temperature for 5 min. After RNase treatment (250 µg/ml), the DNA was stained with propidium iodide (10 µg/ml), and the cell cycle distribution was determined by 10 6 GFP-positive cells with the CellQuest software using a FACScan flow cytometer (Becton Dickinson, Cowley, United Kingdom). For the annexin V assays, the cells were plated on coverslips for 2 d, and then the 3T3 feeder cells were removed before SE treatment. Annexin V-FITC staining was carried out according to the manufacturer’s protocol (CLONTECH, Basingstoke, United Kingdom). The percentage of annexin V-staining cells was then determined microscopically.

In some experiments (e.g., using the Fas/Fc chimeric protein), cells were grown on 3T3 feeders on glass coverslips. Cells were grown to the appropriate density, and the feeder layer was removed and then incubated for an additional 16 h. Cells were then treated with the Fas/Fc chimeric protein (250 ng/ml) for 1 h before treatment with either SE compound or recombinant soluble human Fas-L (150 ng/ml) and the potentiatior antibody (2 µg/ml). After 16-h incubation, the TUNEL assay was performed. Soluble recombinant human Fas-L and mouse monoclonal IgG1 potentiator antibody were obtained from Upstate Biotechnology, Lake Placid, NY; recombinant human Fas/Fc chimera protein was obtained from R & D Systems, Abingdon, United Kingdom.

**Immunoblotting and MAPK Assays.** To prepare whole cell protein extracts for immunoblotting, the cells were washed twice with ice-cold PBS and

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presence of Tris-buffered saline-T [50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA], and 1 mM sodium orthovanadate] and incubated on ice for 20 min, followed by centrifugation at 13,000 rpm in a microfuge for 10 min. The supernatant was stored at −70°C. Up to 50 µg of protein sample in 40 µl of buffer was mixed with 20 µl of loading buffer [187.5 mM Tris-HCl (pH 6.8), 30% glycerol, 6.9% SDS, 2.1 mM ß-mercaptoethanol, and 0.1% bromphenol blue] before electrophoresis on a SDS/8%PAGE gel. The proteins were then blotted onto nitrocellulose (Amersham, Little Chalfont, United Kingdom) using a Camlab semidry blotter, followed by the manufacturer’s protocol. Western blots were preincubated in the loading buffer [187.5 mM Tris-HCl (pH 6.8), 30% glycerol, 6.9% SDS, 2.1 M ß-mercaptoethanol, and 0.1% bromphenol blue] before electrophoresis on a SDS/8%PAGE gel. The proteins were then blotted onto nitrocellulose (Amersham, Little Chalfont, United Kingdom) using a Camlab semidry blotter, following the manufacturer’s protocol. Western blots were preincubated in the presence of Tris-buffered saline-T [50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 0.1% Tween-20 containing 5% dried milk] and then incubated with antibodies diluted in Tris-buffered saline-T containing 5% BSA. Activated or total amounts of specific kinases were measured using the following antibodies: activated JNK monoclonal antibody p-JNK (G-7; Santa Cruz Biotechnology, Santa Cruz, CA); phospho-specific (Ser63/73) c-Jun antibody kit (New England Biolabs, Hitchin, United Kingdom); activated and total p38 kinase, PhosphoPlus p38 MAPK (tyr182) polyclonal antibody kit (New England Biolabs); activated MAPKs (ERKs 1&2), polyclonal antibody (Promega, Southampton, United Kingdom), and total MAPK, ERK 1(2)-16-G polyclonal antibody (Santa Cruz Biotechnology); ERK5 kinase, ERK5(C-20) polyclonal antibody (Santa Cruz Biotechnology); activated Akt and total Akt, PhosphoPlus Akt (ser 473) polyclonal antibody kit (New England Biolabs); and Fas-L, antihuman Fas-L monoclonal antibody (PharMingen/Becton Dickinson, Cowley, United Kingdom). In all cases, bound primary antibody was detected using enhanced chemiluminescence methodology (Amersham). Phosphorylation of ERK5 results in a shift in its electrophoretic mobility detectable by Western blotting and a consistent correlation between the shifted fraction of phosphorylated ERK5 and the activity of ERK5 as measured by protein kinase assay has been observed (data not shown and Ref. 31). This electrophoretic mobility shift was thus used as an assay for ERK5 activity. p38 kinase activity was measured indirectly by a protein kinase assay for the p38 substrate, MAPKAP kinase-2 (32).

Transient Transfection of Cells. HeLa cells were plated at a density of 1.2 × 10^6 cells in 90-mm dishes. The next day, cells were cotransfected with 1 µg each of a GFP construct, pEGFP (CLONETECH), the c-Jun dominant negative construct (TAM67; provided by Prof. B. Ozanne, Beatson Institute, Glasgow, United Kingdom), or a dominant negative FADD-GFP construct (donated by Dr. H. Wajant, University of Stuttgart) using the Effectene (Qiagen, Crawley, United Kingdom) transfection reagent, according to the manufacturer’s protocol. After overnight incubation, fresh medium was added and incubated for another 6 h, before the addition of SE compounds or chemical inhibitors for the appropriate time. Cells were then harvested, and apoptosis assays were performed to assess apoptosis in the GFP-positive population (see above for details).

RESULTS

Sensitivity of NOMCs and SCCs to growth inhibition by SE compounds. Our panel of primary cultures of biopsies of NOMCs and SCCs were derived and maintained using the well-established 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression. The 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression. The 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression. The 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression. The 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression.

We have tested a variety of SE compounds. Our previous studies found that the most effective cancer-protective SE compounds in animal studies, such as selenium, Se-methylselenocysteine, and p-XSC, all induced growth arrest in various cell types, unlike selenomethione, which only affected cell growth at very high concentrations (21). However, whereas most of these SE compounds only inhibit cell growth after a lag period (21), p-XSC induces growth arrest/apoptosis much more quickly, presumably because it is metabolized more rapidly to the directly acting SE derivative. The fact that the lag period required for selenite was reduced by the addition of GSH (33) suggested that the active inhibitor might be SDG, which would be formed by reaction of selenite with GSH either intra or extracellularly, and this was consistent with the fact that purified SDG inhibited cell growth without a significant lag period (33). In the present studies, we therefore focused on elucidating the signal transduction pathways affected by SDG and p-XSC, because their effects were rapid and arguably most likely to be relevant to the chemopreventive effects of the most effective SE compounds, at least in animal models.

Fig. 1 shows the dose-response curves for growth inhibition of NOMCs or SCCs by SDG or p-XSC; to assess whether there were any consistent differences in sensitivity between NOMCs and SCCs, the results for three independently derived NOMCs, or cultures of four carcinoma biopsies, have been pooled. This shows that NOMCs may be less sensitive to SDG than carcinoma cultures in the range 4–5 μM (P < 0.001, using Student’s t test), whereas with p-XSC, any differences are small and not consistent throughout the dose range.

Induction of Apoptosis and Fas-L by SE Compounds. We (21, 34) et al. (20, 22–24) reported previously that SE compounds induce apoptosis in rodent mammary, ovarian, and leukemia established cell lines. We found that both SDG and p-XSC induced apoptosis in oral cell cultures as judged by TUNEL staining, annexin V staining, or measurement of sub-G0 cells by fluorescence-activated cell sorter analysis. Care was taken to ensure that all cultures were subconfluent and growing at their maximum growth rates. Using either TUNEL staining (Fig. 2, A and C) or annexin V staining (Fig. 2, B and D) as assays of apoptosis, SCCs were significantly more sensitive to induction of apoptosis by SDG than NOMCs [Fig. 2, C and D; P = 0.0003 (TUNEL data), P = 0.003 (Annexin data), using the Student’s t test], whereas any differences in sensitivity to p-XSC were marginal and not statistically significant (Fig. 2, C and D; P = 0.39, P = 0.5, respectively). The concentrations of SE compounds required to obtain a significant level of apoptosis were somewhat higher than those required to detect growth arrest by inhibition of DNA synthesis (compare Figs. 1 and 2). All additional molecular studies were performed at SE concentrations found to induce a significant level of apoptosis (usually in the range 20–40%).
V-positive cells (and B and p-XSC induced Fas-L in NOMCs and SCCs under conditions ways were induced by SE compounds, we discovered that both SDG

in NOMCs, whereas p-XSC seemed to be equally effective in both normal cultures (A and B). The results are the averages of two independent NOMCs or carcinoma cultures (± SEs).

In the course of testing whether any of the known apoptotic pathways were induced by SE compounds, we discovered that both SDG and p-XSC induced Fas-L in NOMCs and SCCs under conditions where significant apoptosis was induced (Fig. 3A). Thus, the degree of Fas-L induction corresponded to the secreted form of the protein (35). Fas-L was induced considerably more strongly by SDG in SCCs than in NOMCs, whereas p-XSC seemed to be equally effective in both normal cells and carcinomas (Fig. 3A). Thus, the degree of Fas-L induction correlated closely with the extent of apoptosis (compare Fig. 2). Additional experiments showed that inhibition of the Fas pathway with a Fas/Fc chimera (36) significantly reduced the extent of induction of apoptosis by both SDG and p-XSC (Fig. 3B; P = 0.021, P = 0.044, respectively). Thus, induction of Fas-L is functionally required for induction of apoptosis by these SE compounds.

Effects of SE Compounds on ERKs 1/2, ERK5, and Akt Kinase Signaling Transduction Pathways. We next investigated whether SDG or p-XSC interfered with kinase signal transduction pathways known to control growth or cell survival. ERKs 1&2 (37) and ERK5 (31) have both been implicated in control of cell proliferation by serum mitogens or EGF, whereas the PI-3 kinase/Akt pathway plays an important role in cell survival in a wide variety of cell types (38). ERK5 has also been reported to be redox sensitive and induced by oxidants (39). In view of their increased sensitivity to induction of apoptosis, particularly by SDG, these experiments were performed mainly using SCCs, rather than NOMCs. One way in which SE compounds might induce apoptosis is by interfering with the activation of mitogenic or survival pathways by growth factors. To test whether SDG or p-XSC interfered with activation of the ERKs 1&2 or Akt pathways, SCC cultures were serum starved for 16 h, then pretreated for 2 h with SDG or p-XSC, and then given EGF or serum. Cell extracts were prepared and then analyzed for the extent of activation of ERKs 1&2 or Akt by Western blotting with antibodies that recognize either the total amount of each kinase or only the activated (phosphorylated) forms. These experiments revealed no evidence for a reduction in activation of ERKs 1&2 or Akt by SDG or p-XSC after treatment with EGF or serum (Fig. 4, A and B). Control experiments showed that p-XSC itself induced significant activation of ERKS 1&2, though this was much less marked with SDG (Fig. 4A).

Although it appears paradoxical that an agent causing growth arrest should activate a mitogenic pathway, this has been shown to occur also with other stress factors, such as oxidants (40). However, neither p-XSC nor SDG had any very significant effect on Akt activation (Fig. 4B).

To investigate more thoroughly by functional experiments whether the ERKs 1&2 and Akt pathways were involved in mediating the induction of apoptosis by SE compounds, we tested whether the apoptotic effects of SDG or p-XSC were altered by inhibiting the ERKs 1&2 or Akt pathways with the specific chemical inhibitors, PD98059 (which inhibits activation of ERKs 1&2 by inhibiting MAP extracellular signal-regulated kinase activity) and LY294002 (which inhibits PI-3 kinase and therefore activation of Akt; Ref. 41). Control experiments showed that under the conditions used, PD98059 prevented activation of ERKs 1&2, and LY294002 prevented activation of Akt by SDG and p-XSC (Fig. 5A). However, neither inhibitor protected SCCs from induction of apoptosis by SDG or p-XSC (Fig. 5B); in fact, inhibition of Akt activation by treatment with LY294002 significantly increased the level of SDG-induced apoptosis by ~55% (P < 0.001; Fig. 5B). This might imply that the induction of Akt activation by SDG may be an (inadequate) attempt to protect the cells from apoptosis. These experiments therefore confirm that the ERKs 1&2 and Akt pathways are not of major importance in mediating the induction of apoptosis by either SDG or p-XSC in this oral cell system.

ERK5 is another member of the MAPK family that has also been implicated in control of cell proliferation by EGF in certain cell types (31). We therefore determined whether activation of ERK5 could be detected in our oral cultures by the characteristic change in its migra-

Fig. 2. Sensitivity of NOMCs and SCCs to SE compounds. The degree of apoptosis after treatment of NOMCs or SCCs with solvent control, SDG, or p-XSC for 16 h was quantitated by counting the percentage of TUNEL-positive cells (A and C) or annexin V-positive cells (B and D). The results are the averages of two independent NOMCs or carcinoma cultures (± SEs).

Fig. 3. Induction of Fas-L by SE compounds. In A, NOMCs and SCCs were treated with the stated concentrations of SDG or p-XSC for 16 h, and cell lysates were prepared and Western blotted with either an anti-Fas-L antibody or antibody against total p38 kinase as a loading control. The figures below the blots indicate the fold increase in Fas-L expression above untreated level after SE treatment, normalized to the loading control. B, protection against SDG- and p-XSC-induced apoptosis (measured by the TUNEL assay) after pretreatment of SCCs with a Fas/Fc chimera (250 ng/ml) for 1 h, followed by the indicated concentrations of SDG or p-XSC for 16 h. As a positive control, cells were also treated with soluble Fas-L (sFas-L).

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Control experiments confirmed that 10 ng/ml EGF for 20 min for ERKs 1&2 and ERK5 assays or 10% serum for 30 min for Akt assays. Cell lysates were then prepared and run on SDS-PAGE gels and then Western blotted using antibodies that recognize the total amount of ERKs 1&2 (A) or Akt (B) or only their activated forms (ERKs 1,2* and Akt*). Activation of ERK5 (C) was measured by its change in migration detected by Western blotting with anti-ERK5 antibody (36).

Induction of Stress Kinases by SE Compounds. We next investigated whether the SE compounds induced the JNK and p38 stress kinase pathways because they have been strongly implicated in induction of growth arrest/apoptosis by a wide variety of signals (40). Both SDG and p-XSC caused activation of JNK and p38 in both normal and carcinoma cells under conditions in which apoptosis was induced (Fig. 6). However, SDG was reproducibly more efficient at activating JNK and p38 in SCCs than NOMCs (Fig. 6), thus mirroring the greater sensitivity of SCCs to induction of apoptosis by SDG (Fig. 2).

To test whether the JNK or p38 pathways were functionally important, we performed experiments to determine whether preventing the activation of JNK and/or p38 reduced the extent of apoptosis induced in SCCs by SDG or p-XSC. In the first approach, we exploited the known differential sensitivities of p38 kinase, JNK, and ERKs 1&2 to two chemical inhibitors of the MAPK family, SB202190 and SB203580. Low concentrations (up to ~10 μM) selectively inhibit p38 kinase; at 30 μM, both p38 and JNK are inhibited, whereas ERKs 1&2 are not affected until even higher concentrations (42–44). We therefore tested whether concentrations of SB202190 affected induction of Fas-L and apoptosis by SDG or p-XSC in SCCs. Control experiments confirmed that 10 μM SB202190 prevented SDG- or p-XSC-induced activation of p38 kinase (measured by activation of its downstream kinase MAPKAPK-2 in an immunoprecipitation/kinase assay) but not activation of JNK (measured by phosphorylation of c-Jun at Ser63) or ERKs 1&2 (measured using phospho-specific antibodies), whereas 30 μM SB202190 prevented activation of both p38 kinase and JNK but did not affect activation of ERKs 1&2 (Fig. 7A). Parallel experiments indicated that 30 μM SB202190 prevented SDG and p-XSC induction of Fas-L (Fig. 7B) and apoptosis (for SDG, P = 0.033; for p-XSC, P = 0.041; Fig. 7C), whereas 10 μM SB202190 had no effect (Fig. 7, B and C). This strongly suggests that activation of the JNK pathway is primarily responsible for the induction of Fas-L and apoptosis by SDG and p-XSC.

Using phospho-specific c-Jun antibodies, we found that the SE compounds induced phosphorylation of c-Jun at Ser 63/73, residues known to be phosphorylated by JNK (Fig. 7A and data not shown). Phosphorylation of c-Jun at Ser63/73 is known to be associated with increased transcriptional activity (45), and induction of Fas-L is known to be dependent on c-Jun transcriptional activity (46, 47). Thus, our data provide a plausible explanation as to how induction of JNK by SDG and p-XSC leads to induction of Fas-L and apoptosis. To confirm the functional importance of the JNK/c-Jun pathway directly, we also performed experiments interfering with c-Jun function using a dominant negative mutant of c-Jun, TAM67 (48).

Fig. 5. Effect of treatment of SCCs with the P1-3 kinase/Akt pathway inhibitor (LY294002) or the ERKs 1&2 pathway inhibitor (PD98059) on induction of apoptosis by selenium compounds. A, SCCs were pretreated with 50 μM LY294002 or 50 μM PD98059 and then treated with 8 μM SDG or 30 μM p-XSC for 18 h, and the effect on activation of ERKs 1&2 or Akt was determined by Western blotting using antibodies that recognize phospho-specific or total Akt or ERK. B, induction of apoptosis under the same conditions measured by TUNEL staining as described in Fig. 2.
sufficient numbers, this experiment was performed using HeLa cells because they were found to resemble SCCs in terms of the effects of SDG and p-XSC on JNK, p38, ERKs 1&2, and Akt (data not shown). Thus, a population of HeLa cells expressing TAM67 was obtained by transiently cotransfecting HeLa cells with a GFP vector together with the TAM67 expression vector and analyzing the transfected cells in the GFP-positive fraction by cell sorting (see “Materials and Methods” for details); the transfectants obtained had a level of TAM67 expression at least as high as the endogenous Jun level (Fig. 8A). The level of induction of apoptosis by SDG in TAM67-expressing cells was reduced by 40% (P < 0.001) compared with cells transfected with a vector alone (Fig. 8, B and C). TAM67-expressing cells were only marginally protected (by ~15%) from induction of apoptosis by p-XSC, although this was statistically significant (P = 0.01; Fig. 8, B and C). Experiments were also performed to test whether inhibiting both the JNK and p38 kinase pathways together (by treating TAM67-transfected cells with SB203580 to inhibit p38 kinase activity) was more effective at protecting cells from apoptosis by SDG and p-XSC. However, the extent of protection was identical to that afforded by TAM67 overexpression alone (Fig. 8C). TAM67 expression in HeLa also reduced the level of induction of Fas-L by SDG, but not p-XSC (Fig. 8D), and this correlates with the ability of a dominant negative FADD construct (49) to reduce the extent of apoptosis induced by SDG more than that induced by p-XSC (Fig. 8E), though both reductions were statistically significant (P = 0.014 and P = 0.034, respectively). Overall, these functional transfection experiments with HeLa cells support the conclusion from our data with SCCs based on the use of different concentrations of SB202190 that induction of JNK appears to be the main stress signal transduction pathway functionally required for induction of Fas-L and apoptosis by SDG and p-XSC (although the protection from p-XSC-induced apoptosis by c-Jun inhibition by TAM67 transfection in HeLa cells is not as convincing as that afforded by JNK inhibition by SB202190 in SCCs, for reasons that are presently unclear).

**DISCUSSION**

The main purpose of this work was to identify the signal transduction mechanisms responsible for apoptosis induced by cancer-protective SE compounds using a relevant human model. Our experimental rationale was based on previous work by ourselves and others that SE compounds with the strongest cancer-protective properties in vivo, such as p-XSC, and metabolites of selenite, such as SDG, are also the most effective inducers of growth arrest and apoptosis in vitro.

Our first novel finding is that human SCCs are more sensitive to induction of apoptosis by the SDG than NOMCs. This is clearly of considerable relevance to understanding the cancer-protective effect of SE compounds, particularly selenite. It is not clear why p-XSC does not show the tumor-selectivity exhibited by SDG but may reflect the relative importance of JNK/c-Jun pathway in mediating the effects of the two SE compounds (see below). Since the current work and manuscript were completed, a recent report has also shown that the LNCAp prostate carcinoma cell lines are more sensitive to growth inhibition and induction of apoptosis by selenite or selenomethionine than primary prostate cells, though the molecular signal transduction mechanisms responsible were not explored (50).

The second novel point of interest is that induction of apoptosis by
both SE compounds is associated with a large induction in Fas-L expression above the low basal level in untreated cells; the form of Fas-L produced is the soluble form which could therefore be secreted and act intercellularly. The extent of Fas-L induction by the SE compounds correlates closely with the level of apoptosis induced in normal mucosa or carcinomas, and inhibition of the Fas pathway in SCCs by a Fas/Fc chimera attenuates the induction of apoptosis by SDG and p-XSC. Fas-L expression in biopsies of squamous carcinomas of the head and neck has also been demonstrated recently in another report and shown to be biologically active in inducing apoptosis in cocultivated activated T lymphocytes (51). This is of considerable interest because the Fas-L/Fas receptor interaction is one of the major pathways initiating apoptosis by a variety of agents (46), including alkylating agents (52) and drugs used in chemotherapy (53, 54). Because both NOMCs and SCCs express the Fas receptor constitutively (data not shown), induction of Fas-L may explain why SE compounds induce apoptosis in human oral cells. In vivo, the soluble Fas-L produced by carcinoma cells could also enhance immunological responses that could target the carcinoma cells (55). Activation of Fas pathway by SE compounds could therefore be a factor explaining their effects in enhancing antitumor immune responsiveness (1, 56). Our data show that Fas-L induction by the SE metabolite, SDG, is readily detectable at a SE concentration within the range of plasma concentrations found in humans, although higher than the average level (57). The concentrations of the synthetic SE derivative, p-XSC, required for Fas-L induction are higher than for SDG, but it is well established in animal models that p-XSC has a higher chemopreventive index than selenite, exerting its maximum cancer-protective effect at high dietary levels (about 30 ppm compared with 2 ppm for selenite) because of its lower toxicity (58).

Thirdly, both SDG and p-XSC induce activation of the p38 and JNK stress kinase pathways at concentrations that induce apoptosis, confirming and extending a previous report demonstrating activation of JNK by p-XSC (59). SDG and p-XSC also induce ERKs 1&2 and Akt, but these effects are relatively small (except for the significant effect of p-XSC on ERKs 1&2). Our functional intervention experiments show that activation of the JNK pathway seems to be most important, because inhibition of the JNK pathway, but not the p38 kinase pathway, with appropriate concentrations of SB202190 or using a dominant negative c-Jun construct, reduces SE-induced apoptosis, especially with SDG. Conversely, inhibition of the P-I-3 kinase pathway by LY294002 did not reduce the sensitivity of SCCs to induction of apoptosis by the SE compounds; in fact, it increased the extent of apoptosis induced by SDG, suggesting that the observed induction of Akt in response to SDG was an (unsuccessful) protective response of cells to induction of apoptosis by SDG. Finally, PD98059, a specific inhibitor of ERKs 1&2 activation, did not affect the sensitivity of SCCs to induction of apoptosis by SDG or p-XSC. Moreover, our experiments exclude the possibility that SDG or p-XSC act by inhibiting activation of the ERKs 1&2 or Akt pathways by mitogens, such as EGF or serum which would have biased cells toward cell death.

Induction of JNK by SDG in SCCs is somewhat more marked than in NOMCs, which may therefore explain the increased sensitivity of SCCs to induction of Fas-L/apoptosis by SDG. This effect of micromolar levels of SE in inducing apoptosis via activation of the JNK pathway has to be distinguished from the ability of submicromolar molar levels of SE in inducing apoptosis via activation of the JNK pathway seems to be most important, because inhibition of the JNK pathway, but not the p38 kinase pathway, with appropriate concentrations of SB202190 or using a dominant negative c-Jun construct, reduces SE-induced apoptosis, especially with SDG. Conversely, inhibition of the P-I-3 kinase pathway by LY294002 did not reduce the sensitivity of SCCs to induction of apoptosis by the SE compounds; in fact, it increased the extent of apoptosis induced by SDG, suggesting that the observed induction of Akt in response to SDG was an (unsuccessful) protective response of cells to induction of apoptosis by SDG. Finally, PD98059, a specific inhibitor of ERKs 1&2 activation, did not affect the sensitivity of SCCs to induction of apoptosis by SDG or p-XSC. Moreover, our experiments exclude the possibility that SDG or p-XSC act by inhibiting activation of the ERKs 1&2 or Akt pathways by mitogens, such as EGF or serum which would have biased cells toward cell death.

Induction of JNK by SDG in SCCs is somewhat more marked than in NOMCs, which may therefore explain the increased sensitivity of SCCs to induction of Fas-L/apoptosis by SDG. This effect of micromolar levels of SE in inducing apoptosis via activation of the JNK pathway has to be distinguished from the ability of submicromolar levels of selenite to inhibit UV-induced apoptosis and activation of JNK (60), presumably by increasing the detoxification of reactive oxygen species by increasing the activities of one or more of the known selenoproteins.

The final point of interest is our evidence indicating that induction
of Fas-L by selenium compounds is at least partially dependent on induction of JNK because concentrations of SB202190 that inhibit both JNK and p38 kinase, but not p38 kinase alone, reduce the extent of Fas-L induction in SCCs by SDG and p-XSC, and expression of the dominant negative Jun construct, TAM67, in HeLa cells reduces the induction of Fas-L by SDG. There is other evidence in the literature also indicating that the JNK/c-Jun pathway is mechanistically upstream of Fas-L in other contexts, e.g., after treatment with anticancer drugs or alkylating agents (46, 47). However, this is not necessarily the case (recently reviewed in Ref. 45); e.g., there is evidence that the Fas/Fas-L interaction induces JNK activation, rather than the reverse (61, 62), and that activation of JNK by Fas is not necessarily required for induction of apoptosis (63–65). Fas activation has also been shown to activate both JNK and p38 activation in Jurkat cells; however, in this case, activation of the MKK3/p38 pathway appeared to be important as a downstream target for Fas-induced ICE/CED-3 family proteases (66). Moreover, targeted disruption of FADD, the adapter protein required for Fas-induced apoptosis, does not eliminate stress-induced apoptosis (67). This may be because the Fas death domain of Fas binds independently to two effector molecules: FADD, which couples Fas to procaspase-8, and Daxx, which couples Fas to the JNK pathway (68).

Exactly how SE compounds induce the stress kinase pathways is not yet clear. It has been postulated that SE compounds induce oxidative stress by production of superoxide radicals or hydrogen peroxide (68–70). However, we have previously presented evidence that SE compounds do not induce apoptosis in the same way as hydrogen peroxide (71). More recently, we have shown that the apoptosis-inducing effects of SDG and p-XSC in animal cell lines are mediated in growth control in various contexts and is overexpressed in many tumors (reviewed in Ref. 72). This may be a plausible idea because SDG has been shown to be a specific inhibitor of thioredoxin and thioredoxin reductase in cell-free systems (73, 74). Alternatively, continuously high dietary levels of SE result in a reduction in thioredoxin reductase activity, postulated to be attributable to the formation of an irreversible diselenide “trap” at the selenocysteine in its active site (19); this would also result in a reduction in the level of reduced thioredoxin. Of particular interest in this context is the fact that ASK1, one of the upstream activators of both JNK and p38 (75), is inhibited by binding of reduced thioredoxin to its N-terminal region (76); thus, depletion of reduced thioredoxin by SE compounds by one of the mechanisms described above might be expected to activate ASK1 and induce apoptosis. ASK1 is also regulated by the level of intracellular GSH (77), which may also be affected by the level of SE metabolites. We are currently attempting to test both these hypotheses.

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REFERENCES


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