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Effect of Suramin on Squamous Differentiation and Apoptosis in Three Human Non-Small Cell Lung Cancer Cell Lines

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Abstract Non-small cell lung cancer (NSCLC) is fatal in approximately 90% of all cases due to the failure of systemic therapy, secondary to resistance to chemotherapy. In such malignancies new therapeutic paradigms are needed. One such approach takes advantage of normal physiologic growth regulatory mechanisms, such as terminal cellular differentiation or apoptosis. Suramin, as an antineoplastic drug, has shown efficacy in the treatment of prostate cancer and is capable of promoting differentiation in several human cancer cell lines. Little is known about the differentiating effects of suramin in lung cancer. In the present investigation we evaluated the ability of suramin to induce cross-linked envelope (CLE) formation, as a common marker for squamous differentiation and apoptosis, in three representative human non-small cell lung cancer cell lines: NCI-H226 (squamous), NCI-H358 (bronchoalveolar [adenocarcinoma]), and NCI-H596 (adenosquamous). Among agents that we have tested, suramin demonstrated the unique ability to induce spontaneous CLE formation in the two cell lines with squamous features, NCI-H226 and NCI-H596. Suramin induced CLE formation was accompanied by DNA fragmentation, a marker for apoptosis, in NCI-H596 and NCI-H358, but not in NCI-H226. Stimulation of CLE formation by suramin correlated with the rapid induction of both type II transglutaminase (TG) activity and involucrin expression. These parameters were protein synthesis independent, suggesting posttranslational mechanisms of suramin activity. Induction of differentiation/apoptosis markers by suramin did not correlate with its effect on growth. Modulation of signal transduction is a likely candidate mechanism for suramin activity in lung cancer. The relationship between growth, squamous differentiation, and apoptosis is considered.

Key words: suramin, apoptosis, squamous differentiation, lung cancer

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer death in United States and throughout the world. NSCLC comprises 75–80% of all lung carcinomas [1,2] and is characterized by an exceptionally low cure rate largely due to its resistance to chemotherapy (in contrast to small-cell lung carcinoma, SCLC). The failure of systemic chemotherapy as a modality in NSCLC has stimulated a search for new therapeutic strategies. Paradigms based on the induction of physiological growth regulatory mechanisms, such as terminal cellular differentiation or apoptosis, present a potential alternative to the conventional therapeutic approaches [3,4].

Abbreviations used: CLE, cross-linked envelope; PKC, protein kinase C; TG, transglutaminase.

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NSCLC presents as three major histological types: squamous carcinoma, adenocarcinoma, and large cell undifferentiated [1,2]. Of these forms squamous carcinomas appear to be least aggressive. It has been noted that degree of differentiation inversely correlates with metastatic potential in oral squamous carcinomas [5]. Therefore, understanding mechanisms of induction of squamous differentiation may provide clues for utilizing this process in the treatment of lung cancer. Bronchial epithelial cells can undergo squamous differentiation similar to that normally occurring in keratinocytes [6,7], as a result of squamous metaplasia [8]. The aspect of squamous differentiation best associated with the terminal state, other than karyolysis itself, is formation of the cornified or cross-linked envelope (CLE), comprised of detergent-insoluble covalently cross-linked proteins other than cytokeratins [7–9]. Recent studies have demonstrated

that squamous differentiation may be related to programmed cell death or apoptosis [10]. Apoptotic hepatocytes and neuroblastoma cells can form detergent insoluble highly cross-linked envelopes similar to the classic keratinocyte CLEs by light microscopy [11,12]. The cross-linking process is catalyzed in both processes by the enzyme transglutaminase (TG) although different TG types play role in squamous differentiation and apoptosis [7–13]. Involucrin, a 68 kD protein, is the most abundant cytosolic precursor of the keratinocyte CLEs in humans [14,15]. A protein with immunological identity to involucrin has been demonstrated in apoptotic hepatocytes [16]. Moreover, differentiating keratinocytes undergo DNA fragmentation, which is known to be a characteristic parameter of apoptosis [10,17]. They also show decreasing expression of the oncogene, bcl-2, known to inhibit apoptosis, during progression from the basal to the suprabasal layer [18]. Based on these findings, it has been suggested that squamous differentiation may represent a specialized form of apoptosis [10].

Suramin, a polysulfonated naphthylurea, has been shown to have in vivo and in vitro antitumor effects in several human cancers [19–21]. Part of the antitumor effect of suramin may involve differentiation as has been shown in human colon adenocarcinoma and neuroblastoma cell lines [21,22]. Little is known about the effects of suramin in lung cancer. Suramin has been shown to inhibit proliferation of several NSCLC cell lines [23,24]. There are no reports in the literature on the ability of suramin to induce differentiation or apoptosis in NSCLC cells.

In the present investigation we evaluated the ability of suramin to induce CLE formation in three representative human non-small cell lung cancer cell lines. We have further begun to characterize the mechanism by which these cells enter the terminal phase. Additionally, the relation between induction of programmed cell death and growth regulation is determined. Our findings demonstrate that squamous differentiation/apoptosis are induced, but may be independent of the growth inhibitory mechanism of suramin.

MATERIALS AND METHODS

Cell Culture

Three NSCLC cell lines were used: NCI-H226 (squamous), NCI-H358 (bronchoalveolar [adenocarcinoma]), and NCI-H596 (adenosquamous). The cells were grown in RPMI-1640 media (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL) at 37°C and 5% CO_2. All experiments were performed on heavily confluent cultures (7 days after they reach confluence), in order to potentiate cellular differentiation. Where serum-free conditions were employed, cultures were resupplemented with serum enriched media 24 h prior to starvation followed by 72 h in serum-free media. When incubation with cycloheximide (CHX) was performed, CHX at 20 μg/ml (concentration previously demonstrated by immunoprecipitation to efficiently inhibit TG synthesis [data not shown]) was added 3 h prior to suramin to ensure maximal inhibition of translation.

Growth Assay

Growth was determined based on DNA replication as measured by [H^3]-thymidine incorporation into growing cells. Cells were grown in 24-wells plates and incubated with suramin (Miles, Inc., Elkhart, IN) at different concentrations (50–400 μg/ml) for various time intervals (15 min to 72 h). [H^3]-thymidine [methyl-H^3]thymidine (74.0 Gbq/mmol, 37.0 Mbq/ml) (NEN-Dupont, Boston, MA) was added to the cells at a final concentration of 0.4 μCi/ml. After 1 h of incubation the radioactive media were aspirated, the cells were washed with PBS and precipitated with ice-cold 10% TCA. Cells were then solubilized in 0.25M NaOH and thymidine incorporation was quantified by scintillation counting. Cell counts were performed using hemocytometer, to validate thymidine incorporation in some cases.

Putrescine Uptake

This assay was performed in a manner similar to the growth assay described above, except that [^{3}H]-putrescine hydrochloride (74.0 Gbq/mmol, 37.0 Mbq/ml) (NEN-Dupont) was added at a final concentration of 0.4 μCi/ml rather than thymidine.

Proteinase C (PKC) Activity Assay

Protein Kinase C Assay System was purchased from Gibco BRL and the assay was performed according to the manufacturer's protocol. The assay measures incorporation of ^{32}P in the specific substrate protein during its phosphorylation by PKC. Specific inhibition of phos-

phorylation by pseudosubstrate inhibitor peptide served as negative control.

Differentiation Markers

Cross-linked envelope assay. Induction of CLEs by suramin was performed on confluent cultures grown in 25 cm^2 cell culture flasks (Falcon, Lincoln Park, NJ). Control and treated cells were assayed at a concentration of 10^6 cells/ml. Cells removed by trypsinization were centrifuged in the spent medium in order to recover terminally differentiated cells that may have detached. Each sample was tested for spontaneous, as well as calcium ionophore A23187 (Sigma, St. Louis, MO) induced envelope formation. Incubations with 0.1 mg/ml calcium ionophore were performed for 4 h at 37°C with agitation. Both ionophore-stimulated and spontaneous envelopes were isolated by boiling the cells for 5 min in 2% SDS containing 10 mM DTT [9], and hemocytometer counts were performed in order to quantify the number of CLEs present.

Transglutaminase activity assay. This assay measures enzyme activity by determining the incorporation of [^{3}H]-Putrescine into TCA-precipitable dimethylated casein. Preparation of cytosolic and particulate cell extracts was as previously described [9]. The protein content of each sample was quantified by the method of Bradford using reagents supplied by Bio-Rad Laboratories (Richmond, CA). Cytosolic and particulate TG activity was measured for each sample per microgram of protein. Basic assay conditions were as follows: 200 μl of the assay buffer [9] were added to the samples containing 10 μg of protein extract in 4 ml scintillation vials (Scatron, Sterling, VA) followed by incubation for 30–40 min with agitation. Known concentrations of commercially available guinea pig liver transglutaminase (Sigma) were used for a positive control. The reaction was quenched by precipitation with 20% ice-cold TCA followed by centrifugation at 2,000*g* for 15 min at 4°C. The supernatants were aspirated and samples were washed with 10% TCA followed by centrifugation. After aspiration of the supernatants scintillation fluid was added to each vial and ^{3}H-Putrescine incorporation was determined by liquid scintillation counting.

Cytosolic transglutaminase ELISA assay. Samples containing 10 μg of protein were incubated overnight with 100 μl 50 mM carbonate buffer (pH 9.5) in 96 well immunoplates (Nunc, Naperville, IL). A standard curve was created using known amounts of guinea pig liver TG, with 100 μl of buffer as a blank. Dilution (1:200) of Cub74 (anti-tTG) monoclonal antibody (kindly provided by Dr. Paul Birckbichler) was added to each well. Subsequent steps were performed using a Vector ABC kit according to manufacturer's protocol (Vector Laboratories, Burlingame, CA).

Involucrin ELISA and dot blot analyses. Involucrin ELISA was performed essentially as described above for tTG. Involucrin was assayed in samples containing 2 μg of protein. Polyclonal rabbit anti-involucrin antibody (BTI) was used to quantify involucrin expression. Biotinylated anti-rabbit secondary antibody was employed and involucrin concentrations were assessed using an ELISA plate reader.

For dot blot analysis, samples of 10 μg protein were loaded onto nitrocellulose and analyzed using the same reagents as for ELISA.

DNA fragmentation assay. DNA was isolated from control and suramin treated cells according to the following protocol. Cells were lysed in the buffer containing 0.5% sarcosyl, 10 mM EDTA, 50 mM Tris, pH 8.0, digested with proteinase K (0.5 mg/ml) in total volume of 50 μl at 50°C for 1 h followed by incubation with 10 μl 10 mg/ml of DNAase-free RNAase. Samples were then loaded in dry wells, and DNA was electrophoresed in TAE buffer, visualized under UV-light, and photographed.

Statistics. All experiments were performed in duplicates and mean values were presented ± standard error. Comparisons were performed using one-tailed Student's t test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Suramin on the Proliferation of Human Non-Small Lung Cancer Cell Lines With and Without Squamous Features

In order to determine whether suramin has an antiproliferative effect on NSCLC cell lines and whether this effect is phenotype specific, three cell lines were used: NCI-H596 (adenosquamous), NCI-H358 (bronchioalveolar, lacking squamous features), and NCI-H226 (squamous) [9]. Cells were exposed to two concentrations (50 μg/ml and 200 μg/ml) of suramin for various time intervals. Figure 1 demonstrates the effect of suramin on DNA synthesis in lung cancer cells. Suramin at the above concentrations markedly inhibited ^{3}H-thymidine uptake

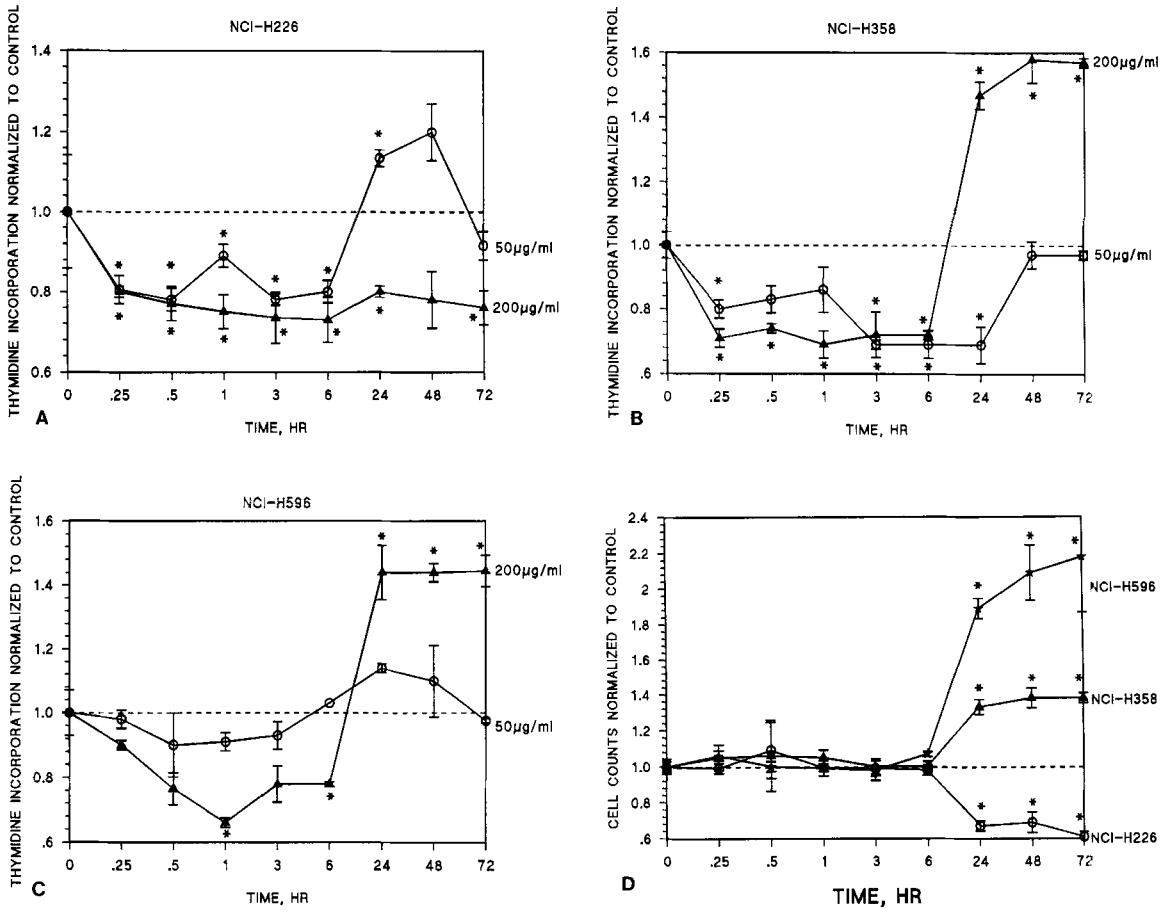

Fig. 1. Suramin effect on DNA synthesis and cell counts in the three NSCLC cell lines. **A–C:** Time- and dose-response curves of [^{3}H]thymidine incorporation with suramin in three NSCLC cell lines. Cells were grown in serum-free RPMI medium where suramin was added. Control wells contained serum-free medium alone. **D:** Time-response curve of cell counts with 200 μg/ml of suramin. For this and other figures determinations were performed at least three times, a representative experiment being presented. Values represent the mean of duplicate determinations ± standard error. Significance of differences between control and experiment at a P value of 0.05 or less is denoted with an *. Statistical analysis was done as described in Materials and Methods.

at the early time points (15 min to 6 h) in all three NSCLC cell lines studied in a dose-dependent manner. Growth suppression occurred under both serum-free and serum supplemented (not shown) conditions. This early effect of suramin had no impact on actual cell proliferation since cell counts did not differ at these time points (Fig. 1D).

Longer incubation of NSCLC cells with suramin (24–72 h) resulted in consistent inhibition of thymidine uptake in NCI-H226 at suramin concentration of 200 μg/ml. In contrast, lower dose of suramin slightly stimulated thymidine incorporation in NCI-H226 under the same conditions (Fig. 1A). The effect of suramin on thymidine incorporation at this time correlated with concomitant changes in cell numbers (Fig. 1D). Surprisingly, in NCI-H358 and NCI-H596, two cell lines demonstrating morphologic features consistent with adenocarcinoma histology, longer incubation with suramin at 200 μl/ml but not at 50 μg/ml, resulted in consistent growth stimulation after 24 h measured by thymidine incorporation (Fig. 1B,C) as well as cell counts (Fig. 1D). Serum supplementation or lack thereof did not alter the effect of suramin on these cell lines (not shown).

Effect of Suramin on Squamous Differentiation/Programmed Cell Death Features in Human NSCLC Cell Lines

CLE formation. We further determined the effect of suramin on spontaneous and calcium ionophore induced CLE formation, as a common

marker for squamous differentiation and apoptosis [10,11]. Suramin at a dose of 200 μg/ml (a concentration consistent with serum levels used in clinical settings [20]) demonstrated the unique ability to induce spontaneous envelope formation in the two cell lines with squamous features, NCI-H226 and 596, as early as 6 h post-addition of drug (Fig. 2A). Of the many agents that we have tested suramin is the first to induce spontaneous CLE in lung cancer cell lines. In agreement with previous observations CLE formation occurred both in the presence (not shown) or absence of serum. This parameter was more sensitive to suramin than was DNA replication, as the effect was seen at relatively low doses (Fig. 2B,C). CLE induction by suramin peaked by 12 h and then reached a plateau. The extent of CLE induction by Ca ionophore without suramin was several fold greater than spontaneous CLE induction by suramin alone. Addition of suramin in the presence of Ca-ionophore further stimulated CLE formation compared to the induction by ionophore only (Fig. 2B,C). The addition of cycloheximide (CHX), an inhibitor of translation, to the incubation media prior to suramin did not abrogate the suramin induced stimulation of CLE in these cells (Fig. 2B,C).

Transglutaminase activity. Stimulation of CLE formation by suramin correlated with the induction of cytosolic TG activity. Suramin had a rapid effect on cytosolic TG activity in NSCLC cell lines; the induction of cytosolic TG activity by suramin was detectable as early as 15 min in NCI-H226 and NCI-H596 with (not shown) or without the serum (Fig. 3A). This induction was dose-dependent (Fig. 3B) and completely disappeared by 6 h. TG in NCI-H358 was also upregulated by suramin, although the level of basal and stimulated activity was extremely low. Suramin had no effect on particulate (membrane bound) TG activity in NCI-H358 and NCI-H596 (not shown), which was very low as compared to cytosolic fraction. However in NCI-H226, where particulate TG was shown to comprise approximately 25% of total TG activity by this assay, suramin stimulated the activity of particulate TG fraction, although to a lesser extent than cytosolic activity (Fig. 3A). The addition of CHX did not abolish the stimulation of cytosolic TG activity in any of the NSCLC cell lines (Fig. 3B).

Effect of suramin on putrescine uptake by cultured cells. In order to determine whether induction of TG activity in NSCLC cells

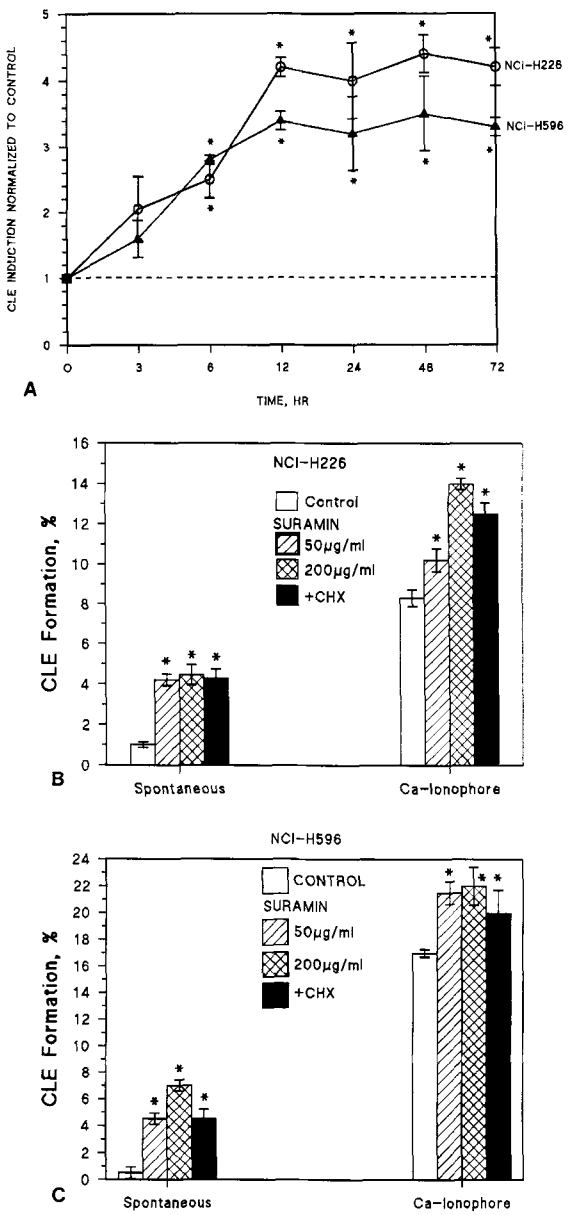

Fig. 2. Induction of cross-linked envelope formation by suramin. **A:** Time-response curve of CLE formation stimulated by suramin (200 μg/ml). **B:** Dose-response curve of CLE induction by suramin in NCI-H226 and **C:** NCI-H596 at 24 h with or without CHX. The differences between Ca-induced CLE formation stimulated by suramin alone or suramin with CHX were statistically insignificant in both cell lines. NCI-H358 did not form envelopes.

by suramin is associated with an increased uptake of polyamines, one of the substrates for TG, we measured the incorporation of radiolabeled putrescine into cultured cells in the presence or absence of suramin. Basal putrescine uptake normalized to cell counts in the three cell lines appeared to be proportional to steady state lev-

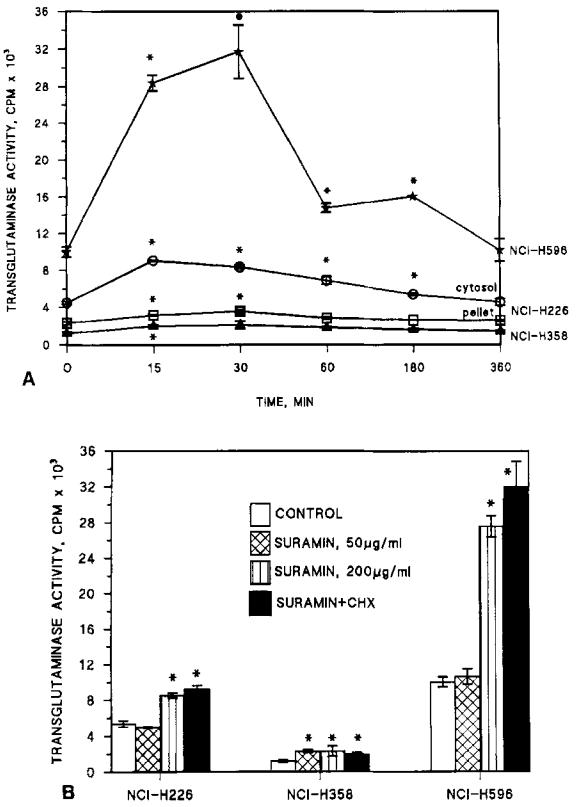

Fig. 3. Effect of suramin on tissue (cytosolic) transglutaminase activity in the three NSCLC cell lines. **A:** Time-response curve with suramin added 200 μg/ml. **B:** Dose curve at 20 min with or without CHX (20 μg/ml). All determinations were made per 10 μg of total protein.

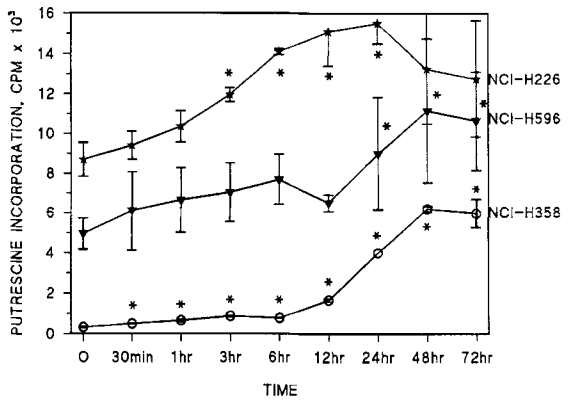

Fig. 4. Suramin-induced [^{3}H]putrescine incorporation in cultured NSCLC cells. Cells were incubated with 200 μg/ml suramin for various time intervals with radiolabeled *putrescine in* serum-free medium.

els of cytosolic TG activity ($r = 0.96$; $P = 0.99$). Addition of suramin promoted a rapid increase of putrescine incorporation into NSCLC cell lines (Fig. 4). The induction was detectable as early as after 30 min of incubation with suramin in NCI-H358 with a sustained increase in putrescine uptake during the period of incubation. By 24 h suramin at a dose of 200 μg/ml stimulated up to 1.7- and 2.1-fold putrescine uptake compared to control in NCI-H226 and NCI-H596, respectively, with NCI-H358 showing more dramatic changes in putrescine uptake with a maximal stimulation of 18.4-fold above control at 48 h.

Tissue transglutaminase ELISA. We did not find detectable changes in immunoreactive type II TG concentration after induction with suramin in any of the three NSCLC cell lines (not shown).

Involucrin concentration. We further determined the effect of suramin on the expression of immunoreactive involucrin, the most abundant human cytosolic protein precursor for human CLEs [14,15]. Suramin upregulated cytosolic involucrin expression in NCI-H226 after 15 min of incubation followed by a marked decrease in immunodetectable cytosolic involucrin by 30 min in both NCI-H226 and NCI-H596 as measured by ELISA and dot blot assays (Fig. 5A,B). By 1 h of incubation with suramin involucrin content was fully restored and by 3 h exceeded the control level in NCI-H226.

Using dot blot analysis which allows to quantify involucrin immunoreactive material in the detergent-insoluble fraction, where, presumably, it is cross-linked, we observed that the decrease in involucrin concentration in cytosol by 30 min was accompanied by simultaneous increase in involucrin in insoluble, but not in particulate, fraction. Involucrin levels in NCI-H358 were very low and did not respond to suramin treatment (data not shown).

DNA fragmentation analysis. Since the predominant form of TG in the three NSCLC cell lines, type II TG, is known to correlate with apoptosis, we determined the presence of a of known marker for apoptosis: nucleosomal DNA fragmentation. We observed DNA ladder formation under the induction by suramin (200 μg/ml) in NCI-H358 and NCI-H596 after 3–6 h of incubation (Fig. 6). Addition of cycloheximide did not affect suramin induced DNA fragmentation (Fig. 6). No detectable DNA cleavage could be found in NCI-H226 under suramin induction, despite its stimulation of CLE formation in this cell line.

Suramin inhibits PKC activity in lung cancer cell lines. Considering the rapidity of suramin induced alterations in NSCLC cells,

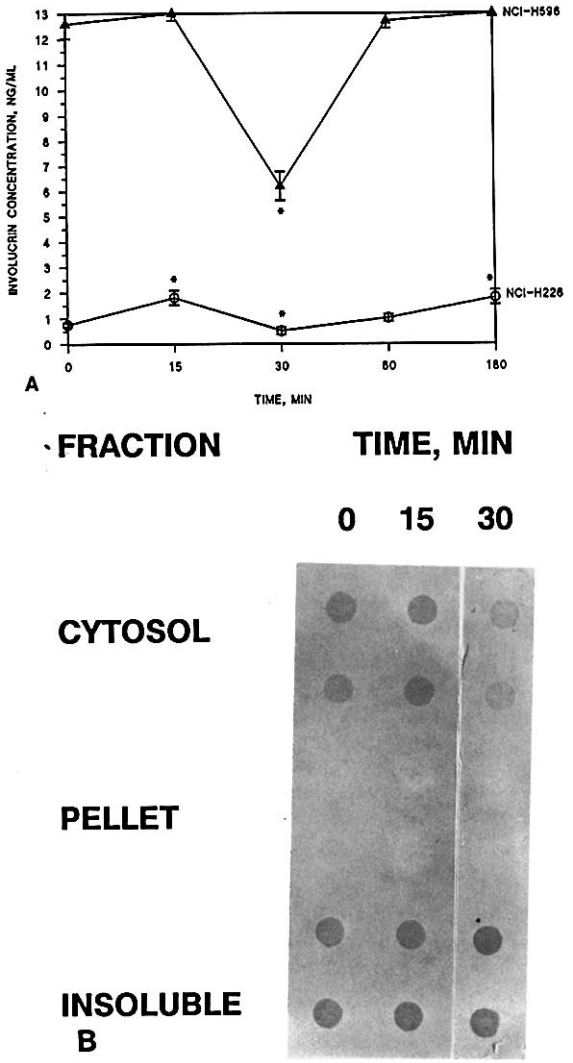

Fig. 5. Effect of suramin on concentration of involucrin in NSCLC cell lines at different time intervals. Cells were incubated with 200 μg/ml of suramin. **A:** Cytosolic involucrin determinations were performed by ELISA as described in Materials and Methods. **B:** Involucrin level in different subcellular fractions of NCI-H596 as assessed by dot blot. Chromogen intensity in the pellet (particulate) fraction was equivalent to that in the non-specific control (not shown). Involucrin levels in NCI-H358 were very low and did not respond to suramin.

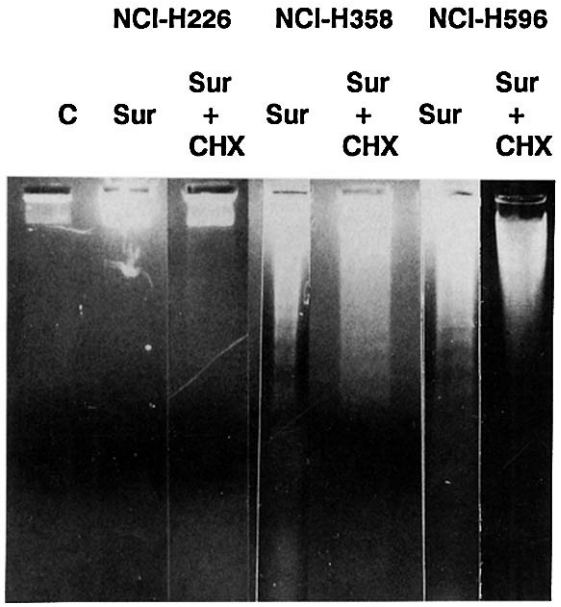

Fig. 6. Demonstration of the presence of fragmented DNA in NSCLC cells by DNA electrophoresis. Suramin was added at a concentration of 200 μg/ml. DNA fragmentation is shown after 24 h of incubation. C, control DNA from untreated NCI-H226. None of the cell lines demonstrated DNA fragmentation when untreated.

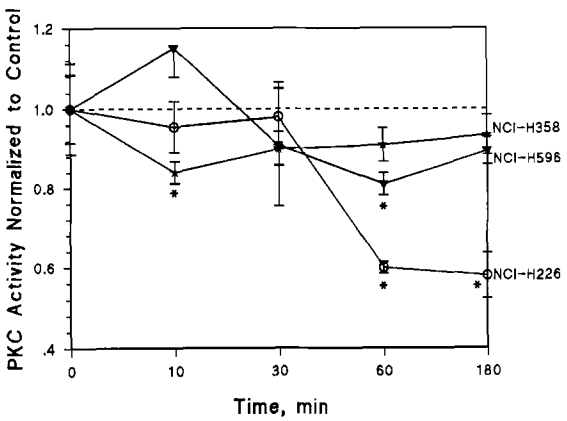

Fig. 7. Total protein kinase C activity in the three NSCLC cell lines with suramin. Cells were incubated with suramin at a concentration of 200 μg/ml for various intervals and PKC activity assayed as described in Materials and Methods.

modulation of signal transduction is a likely candidate mechanism. As shown in Figure 7, suramin at a dose of 200 μg/ml inhibited total protein kinase C activity at 10–60 min post addition in all three NSCLC cell lines. Serum-free media was used for this experiment.

DISCUSSION

Suramin is a polyanionic compound which binds with high affinity to proteins and affects a diverse range of cellular functions. This work presents biochemical and molecular evidence that suramin is capable of affecting growth and terminal squamous differentiation/programmed cell death in three representative NSCLC cell lines.

Suramin is known to affect the growth pattern in many cancerous cell types. Numerous studies have demonstrated that suramin can exert both inhibitory and stimulatory effects on

growth of different cell types [19–25]. This study also demonstrates a differential effect of suramin on growth in three NSCLC cell lines. Suramin at a dose of 200 μg/ml, which is used in clinical applications, induced growth inhibition after 24 h of incubation in a purely squamous cell line, NCI-H226, while clearly stimulating proliferation in two cell lines with adenocarcinoma-type features, NCI-H358 and NCI-H596. Thymidine incorporation was uniformly, although transiently, inhibited after short-term (from 15 min to 6 h) incubation with suramin in all three cell lines. This initial transient inhibition of thymidine incorporation after the addition of suramin did not result in growth inhibition as measured by cell counts. One hypothesis to explain the impact of suramin on growth is based on its ability to bind and neutralize most of the known growth factors. This in turn may have a dual effect: it may result in growth inhibition by blocking growth stimulatory factors, whereas growth stimulation may occur if growth inhibitory factors, such as TGFβ, are neutralized [26]. Although TGFβ has not been shown to inhibit growth in lung cancer cell lines [27], other negative growth factors may exist. In addition one mechanism of growth stimulation may be via suramin induced activation of an autocrine loop involving the receptor for epidermal growth factor (EGFR) [24]. We do not yet know which of the above mechanisms (if any) are responsible for early and late effects of suramin on growth in NSCLC cell lines, or whether these effects are the result of similar or different mechanisms. The ability of suramin to affect thymidine incorporation at as early as 15 min is not surprising, considering the rapidity of other actions of this compound. Suramin has been shown to activate signal transduction pathways. This study, as well as others [28], has demonstrated an inhibitory effect of suramin on the activity of protein kinase C which is known to be involved in proliferation, as well as differentiation and apoptosis [29,30]. Recently the ability of suramin to phosphorylate several cellular proteins after only 10 min of incubation was reported [31]. On the other hand, there is a body of evidence that suramin acts at the nuclear level, directly, indirectly, or both. A possible mechanism for the blocking of DNA synthesis may be via inhibition of DNA pronase, DNA topoisomerase II, and DNA polymerase-alpha [32,33]. However, changes involving nuclear enzymes are unlikely to be responsible for the early suppression of thymidine incorporation by suramin, since they normally occur after at least 24 h of treatment [33]. The rapidity of suramin mediated inhibition of PKC suggests a fairly direct mechanism and precludes those mechanisms that require preceding alterations in the regulation of growth, differentiation, or apoptosis by this drug. Precise mechanisms by which suramin affects growth in NSCLC are still to be elucidated.

Suramin has been shown to induce differentiation in several cancer cell lines: in human colonic adenocarcinoma cells [21] probably through establishment of IGF-II autocrine loop [34], as well as in neuroblastoma cell lines [28]. The differentiating effect of suramin has been attributed to its ability to bind various growth factors, abrogating autocrine loops in a variety of cultured neoplastic cells. In addition there are experimental data suggesting that suramin mediated induction of differentiation may be due to a suramin induced increase in tissue glycosaminoglycans [35,36] or may occur through the inhibition of protein kinase C [28].

The present study is first to report the effect of suramin on apoptosis. We demonstrated that suramin was able to induce formation of spontaneous CLE (as opposed to ionophore induced) in two NSCLC cell lines with squamous features, NCI-H226 and NCI-H596—the first agent that we have identified capable of such. Formation of cross-linked envelopes is a characteristic parameter shared by squamous differentiation and apoptosis [10]. We have further demonstrated that suramin actually induced DNA fragmentation in two NSCLC cell lines, NCI-H358 and NCI-H596, indicative of apoptosis. Interestingly, in the third cell line NCI-H226, the only one of the three cell lines that expressed substantial amount of particulate TG activity and completely lacked any features of glandular differentiation, suramin-induced CLE formation was not accompanied by nucleosomal DNA fragmentation. Additionally, larger fragments (50 kb) were not found by pulse-gel electrophoresis in this cell line (unpublished observation). The inability of NCI-H358 cells to form envelopes despite the induction of DNA fragmentation indicates that apoptosis in NSCLC does not necessarily involve CLE formation. Furthermore, our data regarding redistribution of involucrin between cellular fractions during incubation of cells with suramin indicate that the envelopes formed are not classic keratinocyte

CLE. Thus, it would appear that there are multiple mechanisms by which NSCLC cells may enter a terminal state, induced by suramin. We have previously reported that, in a number of NSCLC cell lines classified morphologically by xenograft histology and ultrastructural studies, CLE competence and prominent expression of transglutaminase and involucrin were associated with the squamous phenotype [9]. While CLE inducibility may be under the control of the squamous phenotype, DNA fragmentation is clearly not, as demonstrated by its occurrence in NCI-H358 in the presence of suramin. We hypothesize that these two events both represent apoptotic programs, and that the squamous metaplastic phenotype in NSCLC represents a form of apoptosis rather than true squamous differentiation.

Stimulation of CLE formation by suramin appears to be dependent upon increased transglutaminase activity, as well as involucrin content, in the two cell lines with squamous features (NCI-H226 and NCI-H596) as the stimulation of TG activity and involucrin precedes CLE formation. As demonstrated by our data, suramin very rapidly upregulated the activity of cytosolic TG in all three NSCLC cell lines. Translocation of involucrin to the insoluble fraction and not to the membrane has been observed which suggests that cross-linking takes place throughout the cytoplasm, rather than submembranously as in keratinocytes [15,37]. Suramin was also able to induce particulate TG activity, which very likely corresponds to type I TG, in NCI-H226. Although we have not yet proven that this transglutaminase is immunologically cross-reactive with anti-type I TG antibody, we have observed that NCI-H226 expresses type I RNA [38]. We have additionally demonstrated the increased uptake of a TG substrate, the primary amine putrescine, in cultured cells in the presence of suramin. Others have reported that polyamine uptake can reflect the level of TG activity [39]. The observed increase in polyamine uptake in our study took place later than the induction of TG activity so that a relationship may be suggested. Based on the time course for stimulation of TG activity, it is also likely that involucrin is initially removed from the cytosolic compartment due to cross-linking by TG.

In NSCLC cells induction of CLEs and DNA fragmentation were protein synthesis independent, since the addition of the translation inhibitor, cycloheximide, did not alter the stimulation of these parameters by suramin. Numerous studies demonstrated that apoptosis in general can not only be independent of protein synthesis, but even be induced by mRNA or protein synthesis inhibitors [40,41]. These data indicate that in these cells effector molecules must be already present within the cell and are regulated by continuous synthesis of specific inhibitors. The fact that CHX itself can induce DNA fragmentation makes it difficult to attribute suramin-induced DNA laddering in NSCLC cells strictly to posttranslational mechanisms. We are currently investigating this issue. CHX, however, had no effect on CLE formation. Thus, it can be concluded that (1) suramin-induced CLE formation was of posttranslational nature, and (2) different pathways lead to fragmentation of DNA and protein cross-linking during apoptosis in NSCLC cells. Surprisingly, activation of TG by suramin also occurred in the presence of CHX. In another experimental system the stimulation of TG activity and CLE competence in NSCLC cells by interferon β were also found not to be CHX sensitive [39]. Using immunoprecipitation we have demonstrated that CHX at 20 μg/ml effectively blocks tissue TG synthesis (data not shown). The most likely interpretation of these observations is that posttranslational modification of tissue transglutaminase is responsible for the altered activity. Several other forms of transglutaminases have been reported to be activated posttranslationally, either by addition of fatty acid residues, as in type I TG [37], or by cleavage of a precursor as in the small epidermal TG [42]. The existence of an inactive form of transglutaminase which, after being cleaved by proteolytic enzymes, gives rise to cytosolic TG, has been reported within metastasizing tumors [43]. Alternatively, transglutaminase activity could be regulated by phosphorylation events [44] or calcium flux, both of which are affected by suramin [28,45]. The ability of cells to activate existing transglutaminases can explain rapid and transient tissue transglutaminase increases accompanying apoptosis/squamous differentiation in NSCLC induced by suramin, and in the rat mesenchymal cells of the limb bud undergoing apoptosis as a result of retinoic acid (RA) treatment [46]. The rapidity of this process may contribute to the very short duration of the histologically visible stages of apoptosis (approximately 3 h) [47]. Studies are underway to elucidate mechanisms by which suramin may induce posttranslational changes in TG activity.

We further questioned whether the regulation of growth and squamous differentiation/apoptosis by suramin occurs via a common pathway, or whether these two processes are independent of one another. In the model proposed by Jetten [8], for normal tracheobronchial cells the first step in the squamous differentiation pathway is growth arrest, followed by increased transglutaminase activity and subsequent CLE formation. In our system stimulation of CLE formation occurred relatively rapidly (3 h). Inhibition of thymidine incorporation was transient in NCI-H596 and did not result in true growth arrest, although TG and CLE formation were stimulated. Based on what is known about the cell cycle length of lung cancer cell lines [48], it is unlikely that growth arrest, even in NCI-H226 where inhibition of thymidine uptake was sustained, could have accounted for the increased CLE formation. On the other hand we can not exclude the possibility that the inhibition of thymidine incorporation observed during the first 3 h of incubation triggered the cells towards the differentiation/apoptosis pathway.

The two cell lines, NCI-H358 and NCI-H596, which display nucleosomal DNA fragmentation characteristic for apoptosis in response to suramin, are also growth stimulated by this agent. This is not surprising in view of the recent studies showing that the cell must be in cycle to enter apoptotic pathway [49] and the same genes control early stages of proliferation and apoptosis [reviewed in 50]. Experimental data indicate that second messengers involved in the pathway of proliferation, such as cAMP, PKC, and tyrosine kinases, can also mediate apoptosis in many cell types [51]. It is likely that the lack or abnormal expression of necessary signals, such as growth factors or second messengers, at the certain check-points of cell cycle can trigger apoptosis [51,52].

The post-translational nature of events induced by suramin suggests that it induces envelope formation in a defined cell population. Thus it is conceivable that suramin may have differential effects on cell growth and death that are subpopulation dependent. One hypothetical determinant of these subpopulations may be cell cycle stage and progression. In fact, it has been demonstrated in several cell types that suramin blocks cells in the S or G2/M phases of the cell cycle [53,54]. Alternatively, the specific response of a particular cell to suramin may depend on the pattern of expression of growth factor receptors or on the configuration of signal transduction pathways.

In conclusion, we have demonstrated that suramin induces squamous differentiation/apoptosis by suramin in NSCLC cell lines. The fact that differentiation/apoptosis appear to be post-translationally regulated suggests that it occurs in a defined population(s) within a given NSCLC cell line. This observation may direct the development of suramin as a therapeutic drug for lung cancer. If the characteristics of the inducible population can be identified, a strategy may be devised that will drive more cells into the differentiation/apoptosis inducible compartment. Such a strategy would develop suramin as an effective chemotherapeutic through a defined mechanism. Development of drugs in this manner allows for the rational planning of combinations. This approach will be enhanced by further understanding of the mechanisms by which CLE formation and DNA fragmentation occur, and how these mechanisms are regulated. Most importantly, the ability of suramin to induce spontaneous CLE and, thus, spontaneous terminal differentiation, in NSCLC, sets it apart from other agents that we have tested, as a model through which squamous differentiation/apoptosis may be developed as therapeutic modalities in this disease.

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