Doxorubicin-induced inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures. Relevance to imparied wound healing.

T Sasaki, … , K C Holeyfield, J Uitto

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Research Article

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Doxorubicin-induced Inhibition of Prolyl Hydroxylation during Collagen Biosynthesis in Human Skin Fibroblast Cultures

Relevance to Impaired Wound Healing

Tetsuo Sasaki, K. Camille Holeyfield, and Jouni Uitto

Department of Medicine, University of California Los Angeles School of Medicine, Division of Dermatology, Harbor-UCLA Medical Center, Torrance, California 90509; and Department of Dermatology and Department of Biochemistry and Molecular Biology, Jefferson Medical College and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract

Previous clinical and experimental observations have indicated that wound healing is impaired as a result of treatment with doxorubicin, a chemotherapeutic agent. In this study, the effects of doxorubicin were examined in human skin fibroblast cultures with respect to collagen production and fibroblast proliferation. The results indicated that the synthesis of hydroxyproline as a marker of collagen production was markedly reduced, with an approximate concentration of inhibitor yielding 50% inhibition of 1 μM. This inhibition could be explained, in part, by generalized inhibition of total protein synthesis, but in addition, there was a significant inhibition of prolyl hydroxylation during collagen biosynthesis, as indicated by a reduction in the ratio of [^{3}H]hydroxyproline/([^{3}H]hydroxyproline + [^{3}H]proline). The latter effect was shown to result from inhibition of prolyl hydroxylase by doxorubicin. As a consequence of reduced prolyl hydroxylation, the stability of newly synthesized procollagen triple helix was shown to be compromised. At the same time, doxorubicin significantly reduced fibroblast proliferation in vitro, as determined by [^{3}H]thymidine incorporation. Thus, reduced collagen production and inhibition of fibroblast proliferation may explain the reduced wound healing in patients undergoing treatment with doxorubicin.

Introduction

Doxorubicin (Adriamycin) is a relatively common chemotherapeutic agent that is used as a treatment modality for a variety of neoplastic diseases (1). The use of doxorubicin is accompanied by several untoward side effects (1), one of them being poor wound healing (2–5). Specifically, clinical observations have demonstrated a significant reduction in wound tear strength in patients administered doxorubicin perioperatively (5). The reduced wound healing in these patients could be explained, at least in part, by the antiproliferative effect of doxorubicin. Animal studies utilizing wound healing models have further demonstrated that collagen production, measured by the synthesis of radioactive hydroxyproline, is markedly reduced in animals given a single dose of doxorubicin on the day of wounding (3). The latter observation, combined with reduced mean collagen fiber diameter (3), suggested that deficient collagen formation may contribute to the reduced wound healing in patients treated with this drug.

In the present study, we have examined the effects of doxorubicin on collagen production in human skin fibroblast cultures. The results indicate that the posttranslational hydroxylation of prolyl residues during collagen biosynthesis is significantly inhibited by doxorubicin. At the same time, doxorubicin markedly reduced the incorporation of [^{3}H]thymidine into cellular macromolecules in rapidly dividing fibroblast cultures. Thus, a dual mechanism, viz. reduced collagen production and inhibition of fibroblast proliferation, may explain the reduced wound healing in patients administered doxorubicin.

Methods

Materials. Doxorubicin hydrochloride (Adriamycin) was purchased from Sigma Chemical Co., St. Louis, MO. In most experiments, doxorubicin was dissolved in DME just before cell culture experiments. In experiments testing the effects of doxorubicin on partially purified prolyl hydroxylase activity in vitro, the test compound was dissolved in 10 mM Tris-HCl, pH 7.5.

Fibroblast cultures. Primary fibroblast cultures were established from normal human skin obtained from cosmetic surgery after informed consent. The primary cultures were passed by trypsinization, and the subcultures were maintained in DME plus glutamine under 5% CO_2-95% air atmosphere in humidified tissue culture incubators at 37°C. The fibroblast cultures were studied in passages 5 and 6.

Collagen assays. For determination of collagen production, 5.0×10^4 cells/well were plated on 2-cm^2 tissue culture plates. In early visual confluency, the incubation medium was replaced by fresh medium containing 0.1 mM ascorbate, 20% dialyzed fetal calf serum and supplemented with doxorubicin in varying concentrations (6). Following a 4-h preincubation, 30 μCi of radioactive proline (L-[2,3,4,5-^{3}H]proline; Amersham Corp., Arlington Heights, IL, sp act, 100 Ci/mmol) was added, and the incubations were continued for 16 h. At the end of the incubation, the medium was removed and stock solutions of proteinase inhibitors were added to yield final concentrations of 10 mM *N*-ethylmaleimide (NEM),[1] 0.3 mM PMSF, and 20 mM EDTA (7). The cells were sonicated in 1.2 ml of 50 mM Tris-HCl, pH 7.5, containing 0.4 M NaCl and the same proteinase inhibitors as above. Aliquots of the media and cell homogenates were dialyzed against running tap water; the samples were hydrolyzed with an equal volume of 12 N HCl for 20 h at 120°C. The total incorporation of ^{3}H radioactivity and the synthesis of [^{3}H]hydroxyproline were then determined (8). Aliquots of the cell homogenate were also used for protein determination by a dye-binding assay (9).

Address correspondence to Jouni Uitto, M.D., Ph.D., Department of Dermatology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107.

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1. *Abbreviations used in this paper:* IC_{50}, concentration of inhibitor yielding 50% inhibition; NEM, *N*-ethylmaleimide.

To determine the degree of prolyl hydroxylation in newly synthesized collagenous polypeptides, fibroblasts in 75-cm^2 tissue culture flasks were incubated with or without 125 μM doxorubicin, and following a 4-h preincubation, 110 μCi of radioactive proline was added. After a 16-h incubation, the medium fraction was recovered and mixed with stock solutions to give final concentrations of 10 mM NEM and 1 mM PMSF. The samples were then dialyzed for 96-h with three changes against 0.4 M NaCl, 50 mM Tris-HCl, pH 7.5, containing 1 mM NEM and 0.1 mM PMSF. 50 μg/ml bacterial collagenase (Sigma type III) and 10 mM $CaCl_2$ were then added, and the samples were incubated for 3 h at 37°C (10). An aliquot of 1.6 ml of each digest was dialyzed against 16 ml of 10 mM acetic acid at 4°C for 72 h. The sample outside the dialysis bag was then lyophilized and hydrolyzed in 6 N HCl for 20 h at 120°C. [^{3}H]Hydroxyproline and [^{3}H]proline were separated by an amino acid analyzer column (PA-28, Beckman Instruments Inc., Fullerton, CA) equilibrated and eluted with a 0.2 M citrate buffer, pH 3.25 at 55°C (11).

Assay of prolyl hydroxylase. To examine the effects of doxorubicin on prolyl hydroxylase activity in fibroblasts, the cells were cultured in 75-cm^2 tissue culture flasks under conditions just described. At the end of a 16-h incubation, medium was removed, the cells were rinsed with HBSS containing 0.2 M NaCl, 50 μM dithiothreitol, 10 μg/ml soybean trypsin inhibitor, and 0.01% Triton X-100 (12, 13). Cell suspension was homogenized with a Teflon–glass tissue homogenizer, stirred gently at 4°C for 60 min, and centrifuged at 4°C at 30,000 *g* for 30 min. Aliquots of the supernatant were dialyzed for 3 h with two changes against 20 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl. Prolyl hydroxylase activity was determined by incubation with [^{3}H]proline-labeled unhydroxylated collagen (protocollagen) substrate, which was prepared from 17-d-old chick embryo leg tendons as described elsewhere (12). The enzyme activity was assayed in the presence of the following final concentrations of cofactors: 2 mM ascorbate, 0.08 mM $FeSO_4$, and 0.5 mM α-ketoglutarate (13). The enzyme incubations were performed at 37°C for 180 min, and the reactions were terminated by the addition of an equal volume of 12 N HCl. The tubes were sealed, hydrolyzed at 120°C for 20 h, and assayed for [^{3}H]hydroxyproline (8).

To examine the effects of doxorubicin on partially purified prolyl hydroxylase, the enzyme was extracted from control fibroblasts incubated on 150-cm^2 tissue culture flasks. The enzyme activity was then determined, as previously described, in the presence of varying concentrations of doxorubicin.

To ensure that the enzyme determinations were performed on the linear range of the assay curve, preliminary control experiments were performed to establish the amount of fibroblast prolyl hydroxylase required to yield linear, dose-dependent synthesis of [^{3}H]hydroxyproline. On the basis of these experiments, an amount of prolyl hydroxylase was used in subsequent experiments, so as to catalyse the synthesis of [^{3}H]hydroxyproline which was in the linear portion of the assay curve. The values for prolyl hydroxylase were expressed as 100 × cpm of [^{3}H]hydroxyproline synthesized per cpm of [^{3}H]proline-labeled protocollagen substrate (%).

Assay of helical stability of procollagen. To test the stability of the triple-helical conformation of newly synthesized procollagens, fibroblasts were cultured on 25-cm^2 tissue culture flasks with 0, 2.5, or 12.5 μM doxorubicin for 4 h, as previously described. Radioactive proline, 50 μCi per flask, was then added, and the incubations were continued for 16 h. The medium was removed, mixed with stock solutions of protease inhibitors, and radioactive procollagens in the media fractions were recovered by precipitation with 176 mg/ml of ammonium sulfate. The precipitate, recovered by centrifugation at 30,000 *g* for 60 min, was dissolved in 0.1 N acetic acid, and pepsin in a final concentration of 300 μg/ml was added. The samples were digested for 6 h at 15°C (7). The digests were examined by SDS-PAGE using 8% gels (14). The radioactive polypeptides were visualized by fluorography (15), and the relative concentrations of α-chains resisting pepsin proteolysis as well as the small molecular weight degradation fragments of radioactively labeled polypeptides were quantitated at 700 nm by a scanning densitometer (Automatic Computing Densitometer, ACD-18; Gelman Sciences, Inc., Ann Arbor, MI).

Other assays. For assay of total protein synthesis, skin fibroblasts on 2-cm^2 tissue culture plates were incubated with doxorubicin in varying concentrations for 4 h, as indicated above; 10 μCi of [^{3}H]leucine was then added. After a 16-h incubation, aliquots of the cell and medium fractions were subjected to precipitation with cold 10% TCA, and the precipitated [^{3}H]leucine-labeled macromolecules were collected on glass fiber filters by a vacuum manifold; the filters were then counted for radioactivity. Another aliquot of the cell homogenate was used for determination of cell protein, and the incorporation of [^{3}H]leucine was expressed as cpm/μg cell protein.

To examine the effect of doxorubicin on fibroblast proliferation, the cells in 96-well microtiter plates were incubated with varying concentrations of doxorubicin for 4 h. Radioactive thymidine ([methyl-^{3}H]thymidine; Amersham Corp., sp act, 84 Ci/mmol), 10 μCi/well, was added and the incubations were continued for 2 h at 37°C. The cells were then harvested with an automated multisample cell harvester (PHD, Cambridge Technology, Inc., Cambridge, MA). The radioactive macromolecules were collected on glass-fiber filters and the radioactivity determined by a liquid scintillation counter.

The viability of the fibroblasts incubated in the presence of varying concentrations of doxorubicin was tested by trypan blue exclusion test (16).

Results

Doxorubicin-induced inhibition of prolyl hydroxylation during procollagen synthesis in skin fibroblast cultures. In the first set of experiments, human skin fibroblasts in confluent monolayer cultures were incubated with [^{3}H]proline in the presence of varying concentrations of doxorubicin, and the synthesis of [^{3}H]hydroxyproline was measured as an index of collagen production; the values were expressed as disintegration-per-minute per microgram cell protein, thus correcting for possible changes in cell number. The results indicated that the synthesis of [^{3}H]hydroxyproline was reduced by doxorubicin in a dose-dependent manner, a 50% inhibition being achieved with ~ 1 μM concentration (Table I and Fig. 1 *A*). This inhibition could

Table I. Effects of Doxorubicin on the Synthesis of [^{3}H]Hydroxyproline and Total Incorporation of ^{3}H Radioactivity in Fibroblast Cultures Incubated with [^{3}H]Proline

Doxorubicin concentration	[^{3}H]Hydroxyproline synthesized	% of control*	Total [^{3}H] incorporation	% of control*
μM	$dpm \times 10^{-3}/\mu g$‡		dpm × $10^{-3}/\mu g$‡	
0	5.07±0.11	100.0	27.97±0.67	100.0
0.2	5.17±0.34	102.0	28.45±0.27	102.0
1	2.21±0.21	43.5	14.28±1.08	51.1
5	1.97±0.03	38.8	15.59±0.87	55.7
25	1.47±0.08	28.9	13.81±0.72	49.4
125	0.45±0.04	8.8	9.57±0.44	34.2

Confluent monolayer cultures of human skin fibroblasts on 2-cm^2 tissue culture plates were preincubated with doxorubicin in concentrations indicated for 4 h. 30 μCi of [^{3}H]proline was then added, the incubations were continued for 16 h, and the synthesis of [^{3}H]hydroxyproline and the total incorporation of ^{3}H radioactivity in the nondialyzable proteins were determined, as indicated in Methods.
* Calculated as percent of the cultures incubated without doxorubicin.
‡ The values are expressed as dpm per μg cell protein (mean±SD of three parallel determinations).

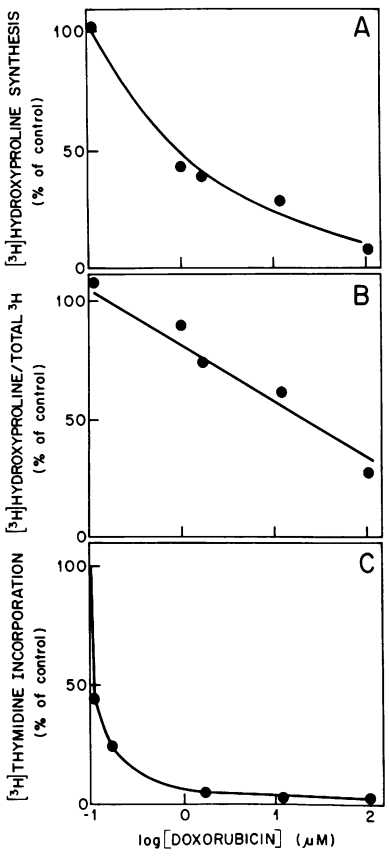

Figure 1. The effects of doxorubicin on the synthesis of [^{3}H]hydroxyproline, degree of prolyl hydroxylation, and the incorporation of [^{3}H]thymidine in human skin fibroblast cultures. (*A*) Human skin fibroblasts in confluent monolayer cultures on 2-cm^2 tissue culture plates were incubated with doxorubicin in concentrations indicated. The synthesis of [^{3}H]hydroxyproline was determined as disintegrations-per-minute per microgram cell protein (see Table I). (*B*) The degree of prolyl hydroxylation in the same cultures as shown in *A* was calculated as 100 × [^{3}H]hydroxyproline/total ^{3}H incorporation (%). (*C*) Skin fibroblasts in 96-well microtiter plates were incubated with doxorubicin for 4 h, and 10 μCi of [^{3}H]thymidine was added. The incubation was continued for 2 h, and the radioactive macromolecules were collected on glass-fiber filters by an automated cell harvester, as indicated in Methods. Values are expressed as percent of control cultures incubated without doxorubicin.

be explained in part by general inhibition of total protein synthesis, because the incorporation of ^{3}H radioactivity ([^{3}H]proline + [^{3}H]hydroxyproline) into nondialyzable proteins was also significantly reduced in cultures incubated with doxorubicin (Table I). Also, a marked inhibition by doxorubicin was noted when [^{3}H]leucine incorporation was used as the marker of total protein synthesis (Table II). To examine the effect of doxorubicin on the posttranslational synthesis of hydroxyproline in detail, the cell cultures were incubated with [^{3}H]proline, and the degree of prolyl hydroxylation was calculated as [^{3}H]hydroxyproline/total ^{3}H incorporated. A dose-dependent inhibition with doxorubicin was noted, and the estimated concentration of inhibitor yielding 50% inhibition (IC_{50}) was ~ 65 μM (Fig. 1 *B*). These observations suggest an inhibition of collagen production, as determined by the synthesis of hydroxyproline, and this inhibition reflects the reduction of collagen synthesis both at pretranslational level as a reflection of the inhibition of total protein synthesis, as well as on the posttranslational level.

To examine the mechanisms of reduced prolyl hydroxylation in cultures incubated with doxorubicin, the newly synthesized collagenous peptides were isolated by digestion with purified bacterial collagenase, and the ratio of [^{3}H]hydroxyproline/[^{3}H]hydroxyproline in these peptides was determined by an amino acid analyzer. In collagen synthesized by the control fibroblasts, the value for 100 × [^{3}H]hydroxyproline/([^{3}H]proline + [^{3}H]proline) was 39.5±0.9%. In cultures incubated with 125 μM doxorubicin, the corresponding value was 17.9% (mean of two separate determinations). Thus, doxorubicin appeared to specifically interfere with the hydroxylation of prolyl residues in newly synthesized collagenous polypeptides. It should be noted that this experiment did not suggest a selective translational inhibition of the synthesis of collagenous polypeptides, because the total reduction in incorporation of ^{3}H radioactivity ([^{3}H]hydroxyproline + [^{3}H]proline) in collagenase-digestable material was the same as the inhibition of total incorporation of ^{3}H radioactivity.

Table II. Effect of Doxorubicin on Total Protein Synthesis in Human Skin Fibroblast Cultures

Doxorubicin concentration	[^{3}H]Leucine incorporation	% of control
μM	dpm/μg*	
0	527.9±111.8	100.0
25	289.3±23.5	54.8
125	39.2±9.6	7.4

Cells in confluent monolayer cultures on 2-cm^2 tissue culture plates were incubated with doxorubicin for 4 h, and 10 μCi of [^{3}H]leucine was then added. After a 16-h incubation, aliquots of the cell and medium fractions were subjected to precipitation with 10% TCA, the precipitated [^{3}H]leucine-labeled macromolecules were collected on glass fiber filters, and counted for radioactivity. Another aliquot of the cell homogenate was used for determination of cell protein.
* Values are dpm per μg cell protein; mean±SD of four parallel cultures.

Doxorubicin inhibits prolyl hydroxylase activity. In additional studies, the mechanism of reduced prolyl hydroxylation was examined in further detail by incubation of the cells with doxorubicin, and the activity of prolyl hydroxylase in the cells was then determined utilizing unhydroxylated collagen (protocollagen) as substrate. The results indicated that the activity of prolyl hydroxylase in cells incubated with 125 μM doxorubicin was reduced to about half of that detected in control cultures (Table III). This observation suggested then that doxorubicin reduced hydroxyproline formation during collagen biosynthesis by inactivating prolyl hydroxylase.

To examine the possibility that doxorubicin would act as a

Table III. The Activity of Prolyl Hydroxylase in Cell Cultures Incubated with Doxorubicin

Doxorubicin concentration	(100 × [^{3}H]hydroxyproline)/total [^{3}H]	% of control
μM	%*	
0	14.98±3.82	100.0
125	8.17±0.36	54.5

Confluent monolayers of human skin fibroblasts in 75-cm^2 tissue culture flasks were incubated with or without doxorubicin for 16 h. Prolyl hydroxylase was then extracted and assayed, as indicated in Methods.
* Values are expressed as percent of [^{3}H]prolyl residues hydroxylated in [^{3}H]proline-labeled protocollagen used as substrate for prolyl hydroxylase. Values are mean±SD of six parallel determinations.

direct inhibitor or inactivator of prolyl hydroxylase, partially purified prolyl hydroxylase was incubated with varying concentrations of doxorubicin in vitro. The results indicated that doxorubicin indeed inhibited prolyl hydroxylase activity, a 50% inhibition being noted with a concentration between 20 and 40 μM (Table IV).

Consequences of reduced prolyl hydroxylation. Because the presence of hydroxyproline plays a critical role in the synthesis and secretion of triple-helical procollagen, it was of interest to test the stability of the triple helix of the newly synthesized [^{3}H]procollagens in cultures incubated with doxorubicin. For this purpose, cells were incubated without or with doxorubicin in 2.5 or 12.5 μM concentration, and labeled for 16 h with [^{3}H]proline. The [^{3}H]proline-labeled procollagen was then subjected to limited pepsin proteolysis using conditions under which the triple-helical collagen domain of type I procollagen resists proteolysis, and the polypeptides can be recovered as intact α-chains in SDS-PAGE.

SDS-PAGE of the digests of procollagen synthesized in control cultures indicated that a large fraction of [^{3}H]proline-labeled procollagen polypeptides was in triple-helical conformation and was recovered after pepsin proteolysis as α-chains (Fig. 2). Also in cultures incubated with 2.5 μM doxorubicin, a large fraction of [^{3}H]procollagen was in a stable triple-helical conformation. In contrast, in cultures incubated with 12.5 μM doxorubicin, very little, if any, intact α-chains were noted (Fig. 2). Scanning densitometry of the fluorograms shown in Fig. 2 indicated that the recovery of the triple-helical α-chains in cultures incubated with 2.5 and 12.5 μM doxorubicin was 70.3 and 24.0% of that in control cultures (Table V). The results suggest that procollagen synthesized in the presence of the latter concentration of doxorubicin was sufficiently deficient in hydroxyproline so as not to fold into a stable triple-helical conformation, as tested by the proteolytic probe. As indicated above, in addition to posttranslational inhibition of prolyl hydroxylation, collagen synthesis was inhibited by doxorubicin on the pretranslational level to the same extent as the protein synthesis in general, and no evidence for selective translational control was observed. Thus, the reduction in collagenous polypeptides which resist proteolysis in doxorubicin-treated cell cultures appears to result from underhydroxylation of collagenous polypeptides, leading to reduced secretion of procollagen molecules with unstable triple-helical conformation.

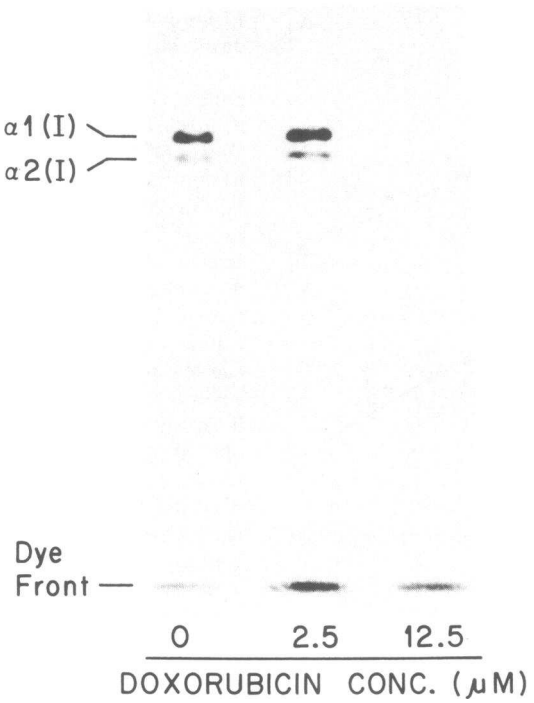

Figure 2. Stability of the triple-helical conformation of [^{3}H]proline-labeled procollagen synthesized in the presence of doxorubicin. Cells in 25-cm^2 tissue culture flasks were incubated with doxorubicin in concentrations indicated. After a 4-h preincubation, [^{3}H]proline was added, the incubations were continued for 16 h, and the newly synthesized procollagens were recovered from the medium by precipitation with ammonium sulfate (176 mg/ml). The newly synthesized procollagens were subjected to limited proteolytic digestion by pepsin, as indicated in Methods. The digestion products were examined by SDS-PAGE using 8% gels, and the radioactive polypeptides were visualized by fluorography. The same amount of radioactivity (2.5 × 10^4 cpm) was electrophoresed on each lane. The migration positions of α1(I) and α2(I) polypeptides of type I collagen, as well as the bromophenol blue dye front are indicated.

Table IV. Inhibition of Prolyl Hydroxylase Activity by Doxorubicin In Vitro

Doxorubicin concentration	(100 × [^{3}H]hydroxyproline)/total [H^3]	% of control
μM	%*	
0	13.67±1.56	100.0
2.5	12.10±2.36	88.5
20.0	10.34±1.38	75.6
40.0	4.94±2.16	36.1

Prolyl hydroxylase was extracted from cultured human skin fibroblasts and assayed in vitro with [^{3}H]proline-labeled unhydroxylated collagen as substrate in the presence of doxorubicin in the concentrations indicated.
* Values indicate the synthesis of [^{3}H]hydroxyproline as percent of total ^{3}H-radioactivity in the substrate (mean±SD of three parallel determinations).

Effects of doxorubicin on proliferation and viability of fibroblasts. Finally, the effects of doxorubicin on the proliferation of skin fibroblasts were determined by measuring the incorporation of [^{3}H]thymidine into cellular macromolecules in actively dividing cultures in logarithmic growth phase. The results indicated a marked, dose-dependent inhibition of DNA synthesis (Fig. 1 *C*); a 50% inhibition of the cell proliferation was noted in the concentration range of ~ 0.1 μM. However, cell viability measured by trypan blue exclusion test after 16 h incubation with doxorubicin revealed no significant difference from the control fibroblasts cultured without doxorubicin. Specifically, the viability of the cells in control cultures was 93.5±1.2% (mean±SD; n = 4), whereas the corresponding values in cultures incubated with 25 and 125 μM doxorubicin were 96.4±2.0 and 96.6±0.9%, respectively.

Discussion

Clinically, one of the recognized side effects of doxorubicin administration is poor wound healing (5). Previous experimental studies utilizing a skin wound-healing model have

Table V. Inhibition of Synthesis of Procollagen in Triple-Helical Conformation by Doxorubicin

Doxorubicin concentration	Medium [^{3}H]protein	Triple-helical [^{3}H]procollagen	Production of [^{3}H]procollagen in triple-helical conformation	% of control
μM	$dpm \times 10^{-5}/10^5$ cells*	fraction of total‡	$dpm \times 10^{-5}/10^5$ cells§	
0	21.4	0.72	15.41	100.0
2.5	17.2	0.63	10.84	70.3
12.5	16.1	0.23	3.70	24.0

Human skin fibroblasts in confluent monolayer cultures in 25-cm^2 tissue culture flasks were incubated with doxorubicin in concentrations indicated. After 4 h preincubation, 50 μCi of [^{3}H]proline was added, and the incubation was continued for 16 h. At the end of the incubation, [^{3}H]procollagens synthesized and secreted into the medium were precipitated with 176 mg/ml of ammonium sulfate. The [^{3}H]proline-labeled proteins were subjected to limited pepsin proteolysis, as indicated in Methods, and the digestion products were examined by SDS-PAGE, as shown in Fig. 2. The ^{3}H-labeled peptides were visualized by fluorography, and their relative distribution determined by scanning densitometry at 700 nm.

* Total [^{3}H]proline-labeled proteins synthesized and secreted into the medium.

‡ Values represent the relative amount of radioactivity detected in α1(I) and α2(I) polypeptides (see Fig. 2) by scanning densitometry as a fraction of total radioactivity in each lane.

§ Calculated by multiplying the total medium [^{3}H]protein (dpm) by the fraction of radioactivity in triple-helical α-chains.

demonstrated that administration of doxorubicin into rats, 6 mg/kg as a single intravenous dose, markedly reduces wound breaking strength when measured at days 14, 21, or 28 after the wounding procedure (2). In a similar study utilizing mice, however, an intraperitoneal administration of doxorubicin, 4 or 10 mg/kg as a single injection, had no effect on wound breaking strength, while injection of 16 mg/kg of doxorubicin significantly impaired wound healing (17). Thus, doxorubicin administration clearly affects the rate of wound healing in experimental animals, although the exact dose required for significant inhibition of wound healing may depend on the animal species and route of administration (4).

Biochemical observations in the rat model have further suggested that impaired collagen synthesis might contribute to poor wound healing in doxorubicin-treated animals. Specifically, the rate of collagen production was reduced in doxorubicin treated animals by > 50% when examined at days 14 and 21 of the healing (3). In the present study, we have examined the potential mechanisms of poor wound healing by incubating cultured human skin fibroblasts with doxorubicin. The results revealed two potential mechanisms which might contribute to poor wound healing. First, doxorubicin markedly inhibited fibroblast proliferation, and the observed IC_{50} of this effect was in the range of 0.1 μM. This concentration is well within the recommended single dosage used for cancer therapy (60–75 mg/m^2) (1). In addition to the inhibition of fibroblast proliferation, a significant reduction in collagen formation, measured by the synthesis of radioactive hydroxyproline, was observed. The observed IC_{50} for inhibition of the synthesis of [^{3}H]hydroxyproline in skin fibroblast cultures was $\sim$ 1 μM. This concentration is also well within the range of the therapeutic dose indicated above. The latter inhibition could be explained by a combination of two separate effects. First, a generalized effect on total protein synthesis was noted, and all evidence suggested that the synthesis of collagenous polypeptides was inhibited to the same extent as the synthesis of noncollagenous proteins. Secondly, an additional, independent effect was clearly detectable on the level of prolyl hydroxylation. Specifically, the ratio of [^{3}H]hydroxyproline/[^{3}H]proline in newly synthesized collagenous polypeptides, isolated by digestion with bacterial collagenase, was markedly reduced. This reduction could be explained by inhibition of prolyl hydroxylase, the enzyme catalyzing the posttranslational hydroxylation of prolyl residues to hydroxyproline (18). The inhibition of prolyl hydroxylation, determined as the ratio of [^{3}H]hydroxyproline/([^{3}H]hydroxyproline + [^{3}H]proline), had an estimated IC_{50} of $\sim$ 65 μM. Although this concentration is higher than the recommended therapeutic dose, two observations suggest that the inhibition of prolyl hydroxylase may significantly add to the overall reduction in collagen biosynthesis. First, our studies demonstrated that incubation of the cells with doxorubicin reduced the helical stability of newly synthesized procollagen, as determined by a proteolytic probe. In fact, in cultures incubated with 12.5 μM doxorubicin the fraction of procollagen resisting pepsin proteolysis, and thus being in a stable triple-helical conformation, was reduced to 24% of that noted in control cultures. Thus, as a consequence of the inhibition of prolyl hydroxylation, the helical stability of newly synthesized procollagen is compromised. Because stable triple-helical conformation is required for normal secretion of procollagen out of the cells (19, 20) and is also required for optimal conversion of procollagen to collagen (21), perturbations in the triple helix could apparently lead to reduced deposition of extracellular collagen fibers. Furthermore, the triple-helical conformation guards against nonspecific proteolysis of the collagenous molecules, and in the absence of a stable triple helix the molecules would be rapidly degraded in the extracellular milieu. Thus, as a result of reduced prolyl hydroxylation, the deposition of collagen in the wound would be reduced.

The second observation relating to the importance of prolyl hydroxylase inhibition is that the synthesis of hydroxyproline in previous experimental models was reduced even after 21 d of a single administration of doxorubicin (3). This observation would suggest that the inhibition of prolyl hydroxylase is irreversible, and this suggestion agrees with recent experimental data obtained with purified prolyl hydroxylase, which indicate that doxorubicin, and a structurally related anthracycline, daunorubicin, act as irreversible inactivators of prolyl hydroxylase (22). It is of interest to note that the IC_{50} for doxorubicin determined by Günzler et al. (22) with purified chick or human prolyl hydroxylase was 60 μM. This concentration is very similar to the value of 65 μM, the concentration of doxorubicin which was shown in our study to reduce the prolyl hydroxylation in cultured fibroblasts by 50%.

Prolyl hydroxylase requires Fe^{2+}, molecular oxygen, α-ketoglutarate, and ascorbate for its activity (for review on prolyl hydroxylase, see reference 18). Prolyl hydroxylase displays an active site that accommodates these small-molecular weight cofactors or cosubstrates (23). Recent observations by

Günzler et al. (22) suggest that doxorubicin acts as a competitive inhibitor with respect to two of the cofactors or cosubstrates of prolyl hydroxylase. Recently, several structural analogues of α-ketoglutarate or ascorbate have been shown to inhibit prolyl hydroxylase (23–25). In fibroblast cultures, the most effective of such structural analogues, ethyl 3,4-dihydroxybenzoate, had an IC_{50} of 0.4 mM (26, 27). Thus, doxorubicin, with an IC_{50} of 1 μM required for inhibition of hydroxyproline synthesis, is a considerably more potent inhibitor of collagen production in fibroblast cultures. It should be noted, however, that the effect of doxorubicin was not limited to collagen, but total protein production as a result of inhibition of DNA replication and inhibition of protein synthesis on the pretranslational level was markedly reduced.

Collagen accumulation is the major pathologic feature in a variety of clinical conditions characterized by tissue fibrosis (28). Progressive systemic sclerosis is the prototype of generalized fibrotic diseases, the excessive accumulation of collagen affecting skin and a variety of internal organs, including the lungs, heart, kidneys and the gastrointestinal tract (29). In several cutaneous diseases, including keloids, hypertrophic scars, familial cutaneous collagenoma, and acquired hamartomas of the collagen type, tissue fibrosis with excessive deposition of collagen is the major pathologic feature (30). In most of these conditions, the excessive tissue deposition of collagen probably results from enhanced production of this protein, because fibroblast cultures established from the lesional skin display enhanced collagen production and elevated procollagen mRNA levels (28). In many of these conditions, increased collagen synthesis may not be the primary event of the disease process, but nevertheless, the excessive accumulation of collagen has major consequences in terms of the structure and function of the affected organs. Thus, a pharmacologic approach that would arrest collagen deposition in the tissues could be beneficial to the patients suffering from clinical fibrotic diseases (25, 31). In this respect, further development of inhibitors or inactivators of prolyl hydroxylase, such as analogues of doxorubicin, might serve as useful agents to limit collagen production in vivo.

In conclusion, results of our study demonstrate that doxorubicin in concentrations well within the range of the therapeutic dose markedly inhibits fibroblast proliferation and impairs the synthesis of collagen as determined by the formation of hydroxyproline. The inhibition of collagen synthesis could be explained by reduction in total protein synthesis, as well as by inhibition of prolyl hydroxylation. Thus, inhibition of fibroblast proliferation and collagen production might well explain the impaired wound healing resulting from doxorubicin administration, as previously demonstrated in clinical and experimental studies (2–5).

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