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Melanin potentiates daunorubicin-induced inhibition of collagen biosynthesis in human skin fibroblasts

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Abstract

One of the recognized side effects of antineoplastic anthracyclines is poor wound healing, resulting from an impairment of collagen biosynthesis. The most affected tissue is skin. The mechanism underlying the tissue specificity of the side effects of anthracyclines has not been established. In view of the fact that a number of pharmacologic agents are known to form complexes with melanin and melanins are abundant constituents of the skin, we determined whether daunorubicin interacts with melanin and how this process affects collagen biosynthesis in cultured human skin fibroblasts. Results indicated that daunorubicin forms complexes with melanin. Scatchard analysis showed that the binding of daunorubicin to melanin was heterogeneous, suggesting the presence of two classes of independent binding sites with $K_1 = 1.83 \times 10^5\ M^{-1}$ and $K_2 = 5.52 \times 10^3\ M^{-1}$. The number of strong binding sites was calculated as $n_1 = 0.158\ \mu mol/mg$ of melanin and the number of weak binding sites as $n_2 = 0.255\ \mu mol/mg$ of melanin. We have suggested that prolidase, an enzyme involved in collagen metabolism, may be one of the targets for anthracycline-induced inhibition of collagen synthesis. We found that daunorubicin induced inhibition of prolidase activity ($IC_{50} = 10\ \mu M$), collagen biosynthesis ($IC_{50} = 70\ \mu M$) and DNA biosynthesis ($IC_{50} = 10\ \mu M$) in human skin fibroblasts. Melanin (100 $\mu g/ml$) by itself produced about 25% inhibition of DNA synthesis and prolidase activity but it had no effect on collagen biosynthesis in cultured fibroblasts. However, the addition of melanin (100 $\mu g/ml$) to daunorubicin-treated cells (at IC_{50} concentration) augmented the inhibitory action of daunorubicin on collagen and DNA biosynthesis without having any effect on prolidase activity. The same effect was achieved when the cells were treated with daunorubicin at one-fourth of the IC_{50} given at 0, 6, 12 and 18 h during a 24-h incubation. The data suggest that the melanin-induced augmentation of the inhibitory effects of daunorubicin on collagen and DNA biosynthesis may result from: (i) accumulation of the drug in the extracellular matrix, (ii) gradual dissociation of the complex, and (iii) constant action of the released drug on cell metabolism. The phenomenon may explain the potential mechanism for the organ specificity of daunorubicin-induced poor wound healing in patients administered this drug. © 2001 Published by Elsevier Science B.V.

Keywords: Melanin; Daunorubicin; Collagen; Prolidase; Fibroblast

1. Introduction

Daunorubicin belongs to the anthracycline class of antitumor agents that has the widest spectrum of activity of all drugs used to treat malignant diseases (Casazza, 1986). The use of anthracyclines is accompanied by several untoward side effects (Calabresi and Parks, 1980). One of them is poor wound healing, especially of the skin. It has been suggested that the antiproliferative effect of daunorubicin (Bland et al., 1984) as well deficient collagen formation (Devereux et al., 1979; Sasaki et al., 1987) may contribute to the reduced healing of skin wounds in patients treated with this drug. However, the precise mechanism underlying the tissue specificity of the daunorubicin-induced reduction of collagen production is not understood.

It is known that several toxins and pharmacologic agents (antibiotics, psychotropic, antirheumatic and anesthetic agents) may undergo a specific interaction with melanin, leading to the accumulation of these agents in melanin-rich tissues and to an increase in their toxicity (Sarna, 1992). The symptoms of their accumulation in melanin-rich tissues may include neuropathy, retinopathy, skin hyperpig-

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mentation and hearing loss (Larsson and Tjälve, 1979; Larsson et al., 1988).

In view of the fact that melanin is an abundant constituent of the skin, it seems reasonable to suspect that the specificity of daunorubicin to inhibit skin collagen biosynthesis may result from its ability to form a complex with melanin, to accumulate in the skin and to have a toxic effect on fibroblasts—the main collagen synthesizing cells.

One of the enzymes involved in collagen biosynthesis is prolidase [E.C.3.4.13.9]. The enzyme is a cytosolic exopeptidase that cleaves imidodipeptides with a C-terminal proline (Endo et al., 1989; Phang and Scriver, 1989). The biological function of the enzyme involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis (Chamson et al., 1989). The efficiency of proline recycling from imidodipeptides is about 90% (Jackson et al., 1975). Therefore, prolidase activity (despite collagen gene expression) may be a limiting factor in the regulation of collagen biosynthesis. Previously, it has been documented that another antineoplastic anthracycline, doxorubicin, inhibits prolidase activity in cultured human skin fibroblasts (Muszyńska et al., 1998), suggesting a mechanism for the anthracycline-induced reduction of collagen biosynthesis.

In the present study, we examined the ability of daunorubicin to form a complex with melanin, the stability constants of this complex and the effect of melanin on daunorubicin-induced inhibition of prolidase activity, DNA and collagen biosynthesis in cultured human skin fibroblasts.

2. Materials and methods

2.1. Materials

Glycyl-proline (Gly-Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma (USA), as were most other chemicals used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum used in cell culture were obtained from Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals (USA). L-5[^{3}H] proline (28 Ci/mmol) was purchased from Amersham (UK). Daunorubicin was a gift from Prof. G. Grynkiewicz (Pharmaceutical Institute, Warsaw). [^{3}H]thymidine (6.7 Ci/mmol) was obtained from NEN (USA).

2.2. Preparation of melanin

Synthetic melanin was obtained by oxidative polymerization of L-DOPA solution (1 mg/ml) in 0.067 M phosphate buffer (pH 8.0) for 48 h, according to Binns et al. (1970).

2.3. Preparation of DNR–melanin complex

Daunorubicin–melanin complexes were obtained as follows: 5 mg of melanin was placed in a plastic test-tube, and drug solutions in bidistilled water were added to a final volume of 5 ml. The initial concentration of daunorubicin ranged from 5×10^{-5} to 1×10^{-3} M. Control samples contained 5 mg of melanin and 5 ml of bidistilled water without drug. All samples were incubated for 24 h at room temperature, and then filtered.

2.4. Analysis of daunorubicin binding to melanin

The concentrations of daunorubicin remaining in each filtrate after incubation with melanin were determined spectrophotometrically, taking the molar absorption coefficient $\varepsilon = 1.1 \times 10^3$ M^{-1} cm^{-1} at 480 nm. All spectrophotometric measurements were made using the UV–VIS spectrophotometer JASCO model V-530. The amount of daunorubicin bound to melanin, calculated as the difference between the initial amounts of drug added to melanin and the amount of unbound drug (in filtrates after incubation) are expressed in μmoles of bound drug per 1 mg melanin.

A qualitative analysis of daunorubicin–melanin interactions was performed using the Scatchard plot of the experimental data according to Kalbitzer and Stehlik (1979). The number of binding sites (n) and the association constant (K) were calculated.

2.5. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from an 11-year-old male donor, were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C under 5% CO_2 in an incubator. The cells were used between the 12th and 14th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in calcium-free phosphate-buffered saline (PBS). For the experiments, cells were counted in hemocytometers and cultured at 1×10^5 cells per well in 2 ml of growth medium in 6-well plates (Costar). Cells reached confluence at day 6 and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug added to the growth medium.

2.6. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium with various concentrations of daunorubicin, melanin or both for 24 h with 5[^{3}H]proline (5 μCi/ml, 28 Ci/mmol) as described previously (Oyamada et al., 1990).

Incorporation of tracer into collagen was determined by digesting proteins with purified *C. histolyticum* collagenase according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

2.7. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. (1982), which is based on the measurement of proline with Chinard's reagent (Chinard, 1952). Briefly, the monolayer was washed three times with 0.15 M NaCl. Cells were scraped off and suspended in 0.15 M NaCl and centrifuged at low speed ($200 \times g$); the supernatant was discarded. The cell pellet (from 2 wells) was suspended in 0.3 ml of 0.05 M Tris–HCl, pH 7.8, and sonicated three times for 10 s at 0°C. Samples were then centrifuged ($18{,}000 \times g$, 30 min) at 4°C. Supernatant was used for protein determination and for the prolidase activity assay. Activation of prolidase requires preincubation with manganese, and therefore 0.1 ml of supernatant was incubated with 0.1 ml of 0.05 M Tris–HCl, pH 7.8 containing 20 mM $MnCl_2$ for 2 h at 37°C. After this preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 94 mM glycyl-proline (Gly-Pro) to a final concentration of 47 mM. After an additional incubation for 1 h at 37°C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes, the reaction was terminated at time "zero" (without incubation). The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by reading absorbance at 515 nm and calculated by using a calibration curve for proline standards. Protein concentration was measured by the method of Lowry et al. (1951). Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

2.8. Cell viability assay

The assay was performed according to the method of Carmichael et al. (1987) using 3-(4,5-di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Confluent cells, cultured for 24 h with various concentrations of studied substances in 6-well plates, were washed three times with phosphate-buffered saline (PBS) and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 M HCl in absolute isopropanol was added to the attached cells. The absorbance of converted dye in living cells was measured at a wavelength of 570 nm with background substraction at 650 nm. The viability of fibroblasts cultured in the presence of daunorubicin, melanin, or both, was calculated as a percentage of that of control cells.

2.9. DNA biosynthesis assay

To examine the effect of the studied substances on fibroblast proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.6 \pm 0.1 \times 10^5$ cells/well), the plates were incubated with various concentrations of melanin or daunorubicin with or without melanin and 0.5 μCi of [^{3}H] thymidine for 24 h at 37°C. Cells were rinsed three times with phosphate-buffered saline (PBS), solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% sodium dodecyl sulfate (SDS), scintillation liquid (9 ml) was added and radioactivity incorporated into DNA was measured in a scintillation counter.

2.10. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (S.D.) were calculated, unless otherwise indicated.

3. Results

The ability of melanin to bind daunorubicin is presented in Fig. 1A. The results demonstrate that the amount of daunorubicin bound to melanin increased with increasing initial drug concentration. It can be seen from the binding isotherm that the amount of daunorubicin bound to the melanin polymer reached a plateau at about 0.3 μmol daunorubicin/mg melanin, which reflects an initial concentration of the drug equal to 6×10^{-4} M. The data were analyzed by constructing a Scatchard plot (Fig. 1B) to determine the binding sites and the number of relevant binding classes. Analysis of daunorubicin–melanin binding showed that the Scatchard plot was curvilinear with an upward concavity, indicating that at least two classes of independent binding sites are implicated in complex formation (Larsson and Tjälve, 1979; Larsson, 1993). The calculated binding parameters for the interaction of daunorubicin with melanin were as follows: strong binding sites with the association constant $K_1 = 1.83 \times 10^5$ M^{-1} and weak binding sites with the association constant $K_2 = 5.52 \times 10^3$ M^{-1}. The number of binding sites were: $n_1 = 0.16$ and $n_2 = 0.25$ μmol daunorubicin/mg melanin.

Confluent human skin fibroblasts were used to test the effect of daunorubicin on prolidase activity and collagen biosynthesis. The rationale for the use of confluent cells in the experiments was that prolidase activity (Myara et al., 1985) and collagen biosynthesis (Makela et al., 1990) are dependent on cell density and increase when the cell

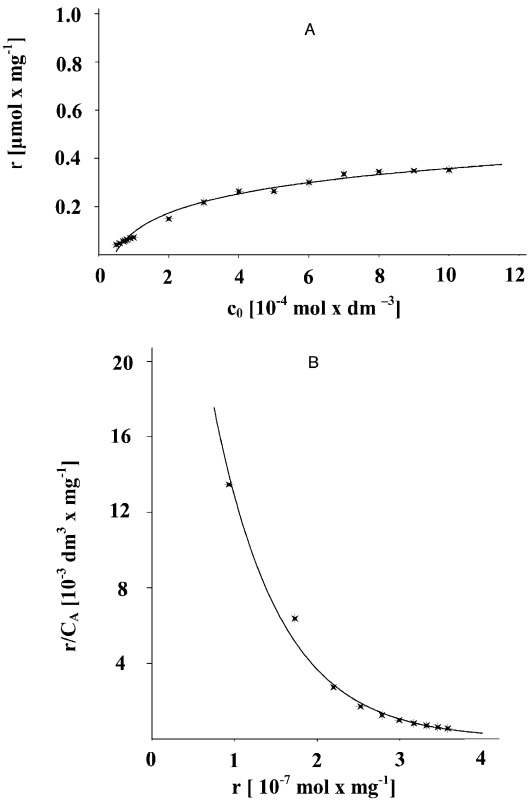

Fig. 1. Binding isotherm (A) and Scatchard plot (B) for the daunorubicin–melanin complex; c_0—initial concentration of daunorubicin, r—amount of daunorubicin bound per 1 mg melanin, c_A—concentration of unbound daunorubicin.

density increases. Prolidase activity and collagen biosynthesis were measured in fibroblasts treated for 24 h with different concentrations of daunorubicin and melanin. The drug decreased collagen biosynthesis in confluent human skin fibroblasts in a dose-dependent manner (Fig. 2). The IC_{50} for collagen biosynthesis was about 70 μM. Melanin had no significant effect on the process.

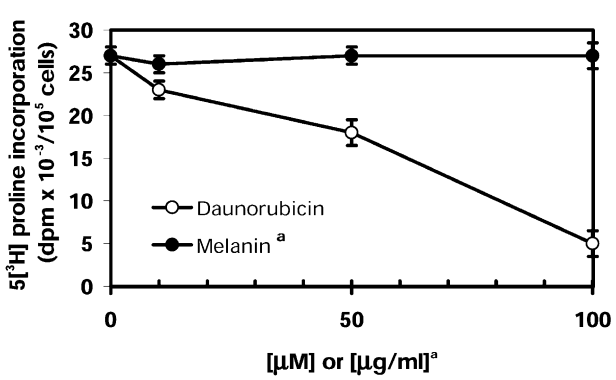

Fig. 2. Collagen biosynthesis (measured as 5[^{3}H]proline incorporation into proteins susceptible to the action of bacterial collagenase) in confluent human skin fibroblasts incubated for 24 h with different concentrations of daunorubicin or melanin. [a]—Melanin concentration was reported as μg/ml of growth medium. Mean values ± S.D. from six independent experiments are presented.

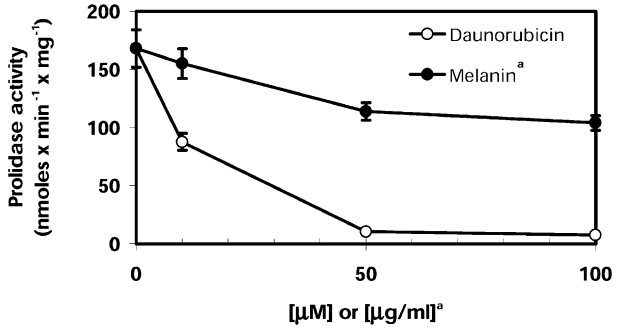

Fig. 3. Prolidase activity in confluent human skin fibroblasts incubated for 24 h with different concentrations of daunorubicin or melanin. [a]—Melanin concentration was reported as μg/ml of growth medium. Mean values ± S.D. from six independent experiments are presented.

A similar effect of daunorubicin on prolidase activity and DNA synthesis was found. The drug decreased fibroblast prolidase activity (Fig. 3) and DNA synthesis (Fig. 4) in a dose-dependent manner. The concentration of the drug required for 50% inhibition (IC_{50}) of prolidase activity was about 10 μM. In both experiments, IC_{50} values were calculated on the basis of the daunorubicin concentration in the medium of cultured cells. Melanin also induced inhibition of both processes. However, at 100 μg/ml of melanin, the processes were decreased only by about 25% of control.

Since daunorubicin was found to form a complex with melanin, we determined the effects of both compounds added simultaneously on prolidase activity, collagen and DNA biosynthesis in confluent fibroblasts. In these experiments, the drug was used at IC_{50} concentrations for the respective processes together with 100 μg/ml of melanin. As can be seen from Fig. 5, the addition of melanin to daunorubicin-treated cells was without an effect on prolidase activity in fibroblasts. In contrast, collagen biosynthesis (Fig. 6) and DNA biosynthesis (Fig. 7) were significantly decreased.

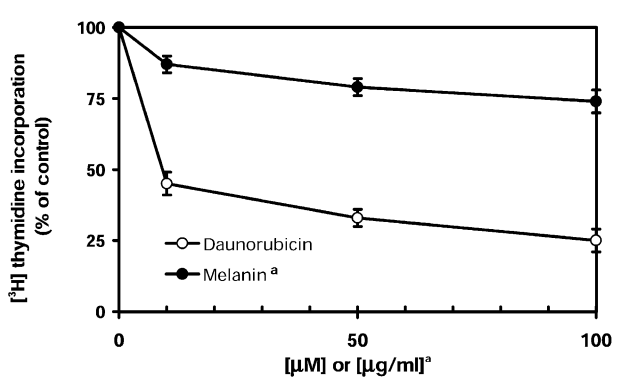

Fig. 4. DNA biosynthesis (measured as [^{3}H]thymidine incorporation into DNA) in semiconfluent human skin fibroblasts incubated for 24 h with different concentrations of daunorubicin or melanin. [a]—Melanin concentration was reported as μg/ml of growth medium. Mean values ± S.D. from six independent experiments are presented.

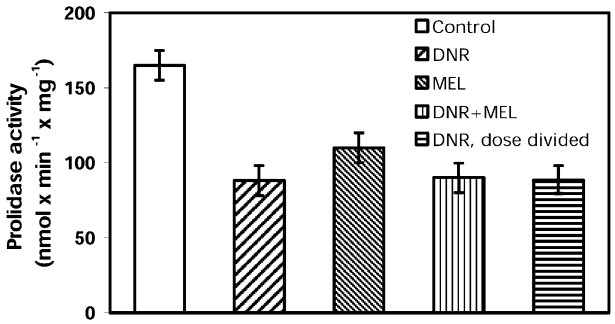

Fig. 5. Prolidase activity in confluent human skin fibroblasts (control) cultured for 24 h with 10 μM daunorubicin (DNR), 100 μg/ml melanin (MEL) or both (DNR + MEL) or with 10 μM daunorubicin given in four doses (2.5 μM) at the start of the experiment and after 6, 12 and 18 h of incubation (DNR, dose divided) in growth medium. Mean values ± S.D. from three independent experiments done in duplicate are presented.

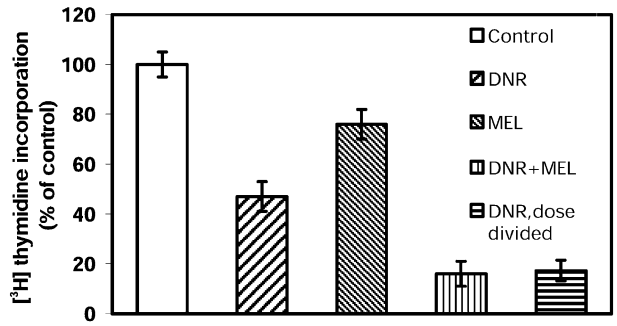

Fig. 7. DNA biosynthesis in semiconfluent human skin fibroblasts (control) cultured for 24 h with 10 μM daunorubicin (DNR), 100 μg/ml melanin (MEL) or both (DNR + MEL) or with 10 μM daunorubicin given in four doses (2.5 μM) at the start of the experiment and after 6, 12 and 18 h of incubation (DNR, dose divided) in growth medium. Mean values ± S.D. from three independent experiments done in duplicate are presented.

In order to elucidate the mechanism underlying this phenomenon, we hypothesized that the daunorubicin–melanin complex formed during the incubation may subsequently dissociate and that the released daunorubicin may constantly affect metabolism. If this were the case, than the addition of the same concentration of daunorubicin (10 μM) to the cells, but divided into several doses given during the time of incubation, would produce similar effects as the daunorubicin–melanin complex. The results presented in Figs. 6 and 7 support this hypothesis. The addition of 10 μM daunorubicin, given in four doses at 0, 6, 12 and 18 h of incubation, produced a similar inhibition of collagen and DNA biosynthesis as the daunorubicin–melanin complex (Figs. 6 and 7). Moreover, daunorubicin added in divided doses caused a stronger inhibition of the processes than the same total concentration of the drug given in one dose. Prolidase activity was not affected either by the daunorubicin–melanin complex or by daunorubicin given in divided doses (Fig. 5).

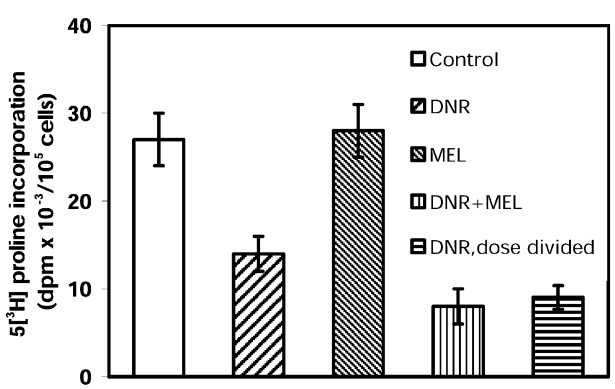

Fig. 6. Collagen biosynthesis in confluent human skin fibroblasts (control) cultured for 24 h with 10 μM daunorubicin (DNR), 100 μg/ml melanin (MEL) or both (DNR + MEL) or with 10 μM daunorubicin given in four doses (2.5 μM) at the start of the experiment and after 6, 12 and 18 h of incubation (DNR, dose divided) in growth medium. Mean values ± S.D. from three independent experiments done in duplicate are presented.

Taking into account that the effects of the studied substances on collagen and DNA biosynthesis may be a result of drug cytotoxicity, we assessed cell viability after addition of different concentrations of daunorubicin, melanin, or both. Cell viability was measured by the method of Carmichael et al. (1987) using tetrazolinum salt. Because of the possibility that daunorubicin interacts with the tetrazolinum salt, the control test (non-specific reaction) was performed with both reagents incubated for 24 h in cell-free system. Values were corrected for this non-specific reaction. The viability of cells incubated for 24 h with the indicated concentrations of daunorubicin is presented in Table 1. As can be seen, daunorubicin at a concentration of 50 μM (the concentration at which collagen and DNA biosynthesis were strongly inhibited) pro-

Table 1
Viability of confluent human skin fibroblasts treated with different concentrations of the indicated substances
Mean values ± S.D. from six independent experiments are presented.

Substance: Daunorubicin (μmol/l)	Cell viability (% of control)
0	100
10	92.69 ± 3.3
50	83.2 ± 4.1
100	53.3 ± 3.1
Substance: Melanin (μg/ml)	**Cell viability (% of control)**
0	100
10	99.5 ± 0.4
50	98.2 ± 0.7
100	95.2 ± 1.3
Substance: Daunorubicin + melanin[a] (μmol/l) or (μg/ml)[a]	**Cell viability (% of control)**
0	100
10 + 100[a]	97.2 ± 1.8
50 + 100[a]	89.6 ± 2.6
100 + 100[a]	83.0 ± 4.3

[a] Melanin concentration reported as μg/ml of growth medium.

duced only about a 17% reduction of the viability of these cells. This suggests that the strong inhibitory effect of daunorubicin (at 50 μM) on collagen and DNA biosynthesis cannot be exclusively due to the cytotoxicity of this drug at the indicated concentrations. Melanin, even at a high concentration (100 μg/ml), produced only about a 5% reduction in cell viability. The addition of melanin (100 μg/ml) to daunorubicin-treated cells (at all indicated concentrations) significantly decreased the cytotoxicity of daunorubicin. This suggests that the melanin-induced potentiation of the daunorubicin-dependent inhibition of collagen and DNA synthesis is not a result of increased cytotoxicity.

4. Discussion

One of the recognized side effects of antineoplastic anthracyclines is poor wound healing, resulting from an impairment of collagen biosynthesis. The most affected tissue is skin. The mechanism for the organ specificity of the side effect of anthracyclines has not been established. In view of the fact that a number of pharmacologic agents are known to form complexes with melanins and melanin is abundant constituent of the skin, we determined whether daunorubicin interacts with melanin and how this process affects collagen biosynthesis in cultured fibroblasts. The data presented here show that melanin forms complexes with daunorubicin and that at least two classes of independent binding sites are implicated in complex formation. The two calculated binding sites in the daunorubicin–melanin complex may be due to a different accessibility of the melanin binding sites. This may be explained by differences between the surface and the interior of the melanin polymer with regard to steric hindrance and physico-chemical conditions, as was earlier suggested (Larsson and Tjälve, 1979; Larsson, 1993). Melanins are polyanions with a relatively high content of carboxy groups and *o*-semiquinones (Ito, 1986; Prota, 1992), which are negatively charged at physiological pH. Substances with cationic properties (e.g., metal ions, some drugs) are thus bound to melanin mainly by ionic interaction, which also may be strengthened by other forces such as van der Waals attraction, charge-transfer reactions and hydrophobic interactions (Larsson and Tjälve, 1979; Larsson et al., 1988).

The ability of melanin to bind daunorubicin was accompanied by the melanin-dependent potentiation of the inhibitory action of daunorubicin on collagen and DNA biosynthesis. One possible explanation for this phenomenon is that the daunorubicin–melanin complex (which had a relatively low stability constant) gradually dissociates during the 24-h incubation of cultured cells and that the released daunorubicin constantly affects the metabolism of the cells. Although 10 μM daunorubicin produced about 50% reduction in DNA synthesis in fibroblasts during a 24-h incubation, the same total concentration (10 μM) divided into four doses (2.5 μM each) added to the growth medium at the start of the incubation and 6, 12 and 18 h later produced about 80% inhibition of the process after 24 h of incubation. A similar effect was found with respect to collagen synthesis. The data suggest that the mechanism of the melanin-dependent potentiation of the effects of daunorubicin on cell metabolism involves: (i) the ability of melanin to form a complex with daunorubicin, (ii) the accumulation of the drug in the extracellular matrix, and (iii) the gradual release of daunorubicin from the complex so that it exerts a constant inhibitory action on collagen and DNA biosynthesis. Since melanin is an abundant constituent of the skin, the phenomenon may explain the organ specificity of the poor wound healing seen in patients administered this drug.

Another interesting point arises from the data, namely, whether the melanin–daunorubicin complex serves as a pro-drug with a prolonged duration of action. If this is true, one would expect that similar therapeutic effects of daunorubicin would be achieved with lower doses of the melanin–daunorubicin complex.

Although melanin potentiated the inhibitory effect of daunorubicin on collagen biosynthesis, it had no effect on the potentiation by daunorubicin of prolidase activity in fibroblasts. The biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis (Chamson et al., 1989; Jackson et al., 1975; Yaron and Naider, 1993). It is evident that an absence of prolidase will severely impede the efficient recycling of collagen proline (Goodman et al., 1968). However, enhanced liver prolidase activity was found during the fibrotic process (Myara et al., 1987). This suggests that prolidase, by providing proline for collagen biosynthesis, may regulate the turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Recently, collagen production and prolidase activity were found to be associated in cultured human skin fibroblasts treated with anti-inflammatory drugs (Miltyk et al., 1996), during experimental aging of these cells (Palka et al., 1996), fibroblast chemotaxis (Palka et al., 1997) and cell-surface integrin receptor ligation (Palka and Phang, 1997). Our results confirmed the previous observation of anthracycline-dependent inhibition of prolidase activity and collagen biosynthesis in cultured fibroblasts (Muszyńska et al., 1998). However, unlike collagen biosynthesis, the inhibition of prolidase activity by daunorubicin was not augmented or affected by melanin. The discrepancy between the melanin-dependent potentiation of the inhibitory effect of daunorubicin on collagen biosynthesis and the lack of an effect on prolidase activity is being intensively studied in our laboratory.

The data suggest that the ability of daunorubicin to form a complex with melanin may contribute to the accumulation of the drug in melanin-rich tissues (e.g., epidermal tissue), with the subsequent slow dissociation of the

complex, leading to the release of daunorubicin and augmentation of its inhibitory action on collagen and DNA biosynthesis in dermal cells. The phenomenon may explain the organ specificity of the poor wound healing seen in patients administered this drug.

References

Binns, F., Chapman, R.F., Robson, N.C., Swan, G.A., Waggot, A., 1970. Studies related to the chemistry of melanins: Part VIII. The pyrrole-carboxylic acids formed by oxidation or hydrolysis of melanins derived from 3,4-dihydroxyphenethylamine or 3,4-dihydroxyphenyl-alanine. J. Chem. Soc. 1128–1134.

Bland, K.I., Palin, W.E., Fraunhofer, J.A., Morris, R.R., Adcock, R.A., Tobin II, G.R., 1984. Experimental and clinical observations of the effects of cytotoxic chemotherapeutic drugs on wound healing. Ann. Surg. 199, 782–790.

Calabresi, P., Parks Jr., R.E., 1980. Chemotherapy of neoplastic diseases. In: Gilman, A.G., Goodman, L.S., Gilman, A. (Eds.), The Pharmacological Basis of Therapeutics. Macmillan, New York, pp. 1249–1313.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., Mitchell, J.B., 1987. Evaluation of a tetrazolinum-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. 47, 936–942.

Casazza, A.M., 1986. Preclinical selection of new anthracyclines. Cancer Treat. Rep. 70, 43–49.

Chamson, A., Voigtlander, V., Myara, I., Frey, J., 1989. Collagen biosynthetic anomalies in prolidase deficiency: effect of glycyl-L-proline on the degradation of newly synthesized collagen. Clin. Physiol. Biochem. 7, 128–136.

Chinard, F.P., 1952. Photometric estimation of proline and ornithine. J. Biol. Chem. 199, 91–95.

Devereux, D.F., Thibault, L., Boretos, J., Brennan, M.F., 1979. The quantitative and qualitative impairment of wound healing by adriamycin. Cancer (Philadelphia) 43, 932–938.

Endo, F., Tanoue, A., Nakai, H., Hata, A., Indo, Y., Titani, K., Matsuda, I., 1989. Primary structure and gene localization of human prolidase. J. Biol. Chem. 264, 4476–4481.

Goodman, S.I., Solomons, C.C., Muschenheim, F., Macintyre, C.A., Miles, B., O'Brien, D., 1968. A syndrome resembling lathyrism associated with iminodipeptiduria. Am. J. Med. 45, 152–159.

Ito, S., 1986. Reexamination of the structure of eumelanin. Biochim. Biophys. Acta 883, 155–161.

Jackson, S.H., Dennis, A.N., Greenberg, M., 1975. Iminopeptiduria. A genetic defect in recycling collagen: a method for determining prolidase in red blood cells. Can. Med. Assoc. J. 113, 759–763.

Kalbitzer, H.R., Stehlik, D., 1979. On the analysis of competitive binding of various ligands to cooperative and independent binding sites of macromolecules. Z. Naturforsch. 34c, 757–769.

Larsson, B.S., 1993. Interaction between chemicals and melanin. Pigm. Cell Res. 6, 127–133.

Larsson, B.S., Tjälve, H., 1979. Studies on the mechanism of drug-binding to melanin. Biochem. Pharmacol. 28, 1181–1187.

Larsson, P., Larsson, B.S., Tjälve, H., 1988. Binding of aflatoxin B_1 to melanin. Food Chem. Toxicol. 26, 579–586.

Lowry, O.H., Rosebrough, N.I., Farr, A.L., Randall, I.R., 1951. Protein measurement with the Folin reagent. J. Biol. Chem. 265, 193.

Makela, J.K., Vuorio, T., Vuorio, E., 1990. Growth-dependent modulation of type I collagen production and mRNA levels in cultured human skin fibroblasts. Biochim. Biophys. Acta 1049, 171–176.

Miltyk, W., Karna, E., Pałka, J., 1996. Inhibition of prolidase activity by non-steroid antiinflammatory drugs in cultured human skin fibroblasts. Pol. J. Pharmacol. 48, 609–613.

Muszyńska, A., Pałka, J., Wołczyński, S., 1998. Doxorubicin-induced inhibition of prolidase activity in human skin fibroblasts and its implication to impaired collagen biosynthesis. Pol. J. Pharmacol. 50, 151–157.

Myara, I., Charpentier, C., Lemonnier, A., 1982. Optimal conditions for prolidase assay by proline colorimetric determination: application to imidodipeptiduria. Clin. Chim. Acta 125, 193–205.

Myara, I., Charpentier, C., Gantier, M., Lemonnier, A., 1985. A cell density affects prolidase and prolinase activity and intracellular amino acid levels in cultured human cells. Clin. Chim. Acta 150, 1–9.

Myara, I., Miech, G., Fabre, M., Mangeot, M., Lemonnier, A., 1987. Changes in prolinase and prolidase activity during CCl_4 administration inducing liver cytolysis and fibrosis in rat. Br. J. Exp. Pathol. 68, 7–13.

Oyamada, I., Palka, J., Schalk, E.M., Takeda, K., Peterkofsky, B., 1990. Scorbutic and fasted guinea pig sera contain an insulin-like growth factor-I reversible inhibitor of proteoglycan and collagen synthesis in chick embryo chondrocytes and adult human skin fibroblasts. Arch. Biochem. Biophys. 276, 85–93.

Pałka, J.A., Phang, J., 1997. Prolidase activity in fibroblasts is regulated by interaction of extracellular matrix with cell surface integrin receptors. J. Cell. Biochem. 67, 166–175.

Pałka, J., Miltyk, W., Karna, E., Wołczyński, S., 1996. Modulation of prolidase activity during in vitro aging of human skin fibroblasts the role of extracellular matrix collagen. Tokai J. Exp. Clin. Med. 21 207–213.

Pałka, J.A., Karna, E., Miltyk, W., 1997. Fibroblast chemotaxis and prolidase activity modulation by insulin-like growth factor II and mannose 6-phosphate. Mol. Cell. Biochem. 168, 177–183.

Peterkofsky, B., Chojkier, M., Bateman, J., 1982. Determination of collagen synthesis in tissue and cell culture system. In: Furthmay, M.M. (Ed.), Immunochemistry of the Extracellular Matrix. CRC Press, Boca Raton, FL, pp. 19–47.

Phang, J.M., Scriver, C.R., 1989. Disorders of proline and hydroxyproline metabolism. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic Basis of Inherited Disease. McGraw-Hill, New York, pp. 577–597.

Prota, G., 1992. Melanin and Melanogenesis. Academic Press, New York, 290 pp.

Sarna, T., 1992. Properties and function of the ocular melanin. A photobiophysical view. J. Photochem. Photobiol. 12, 215–258.

Sasaki, T., Holeyfield, K.C., Vitto, J., 1987. Doxorubicin-induced inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures. J. Clin. Invest. 80, 1735–1741.

Yaron, A., Naider, F., 1993. Proline-dependent structural and biological properties of peptides and proteins. Crit. Rev. Biochem. Mol. Biol. 28, 31–81.