Anti-Cancer Drugs 1994, **5**, pp. 99–104

Effects of 5-fluorouracil on collagen synthesis in the developing palate of hamster

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A study was undertaken to examine the effect of 5-fluorouracil (5-Fu) on collagen synthesis in the developing secondary palate. Pregnant hamsters were given 81 mg/kg 5-FU intramuscularly or 1 ml saline on day 11 of gestation. Control and treated embryonic palates, dissected from hamsters between days 11 and 13 of gestation, were incubated in a growth medium supplemented with [^{14}C]proline. The rate of collagen synthesis, total protein and collagen isotypes were determined. The data showed that in control hamster palate the collagen synthesis peaked between days 12:00 (12 day: 0 h) and 12:04 of gestation, which is the period of shelf reorientation. In 5-FU-exposed hamster palates, the rate of collagen synthesis was lower than controls until day 12:04 of gestation followed by a rise on day 12:12 of gestation. In 5-FU-treated embryos palatal shelf reorientation took place between days 12:16 and 13:00 of gestation. Electrophoresis showed that only type I collagen was synthesized during palate development in both the control and 5-FU-treated hamster embryos. It was suggested that collagen synthesis may play a critical role in shelf reorientation in hamster since (i) new collagen was synthesized in both the control and 5-FU-treated hamster embryos prior to and during the period of normal reorientation, and (ii) in 5-FU-treated hamster embryos, a recovery in collagen synthesis precedes reorientation. Inhibition of collagen synthesis during abnormal development is only a step in the cascade of events of 5-FU-induced effects on protein synthesis.

Key words: Collagen synthesis, developing palate, 5-fluorouracil, hamster.

Introduction

The antineoplastic agent, 5-fluorouracil (5-Fu), was synthesized in 1957.[1] In humans the drug is mainly used in the treatment of various carcinomas.[2,3] In addition, 5-FU is also teratogenic in laboratory animals and humans causing malformations of brain, eye, lung, kidney, mandible, palate and limb.[4–9] The drug crosses the placental barrier rapidly and reaches the fetus within 30 min.[10] Although the mechanism by which 5-FU exerts its anti-neoplastic or teratogenic effects is unclear, it is suggested that 5-FU may inhibit production of ribosomal RNA which in turn could depress protein synthesis.[3,11–13]

In the present study, we have used the developing secondary palate of hamster as a system to elaborate on the mechanism of 5-FU action. The hamster palate initially develops as two vertical shelves alongside the tongue. The shelves then reorient from a vertical to a horizontal plane, dorsal to the tongue, and unite with each other to separate the oral and nasal cavities.[14] Administration of 5-FU delays reorientation of palatal shelves by approximately 16 h to induce a cleft palate.[15–17] One of the important aspects of shelf reorientation during development is the synthesis of extracellular matrices (ECM), mainly glycosaminoglycans (GAG) and collagen.[18–24] Recently, we have examined the effects of 5-FU on GAG synthesis, DNA synthesis and tissue morphology.[16,17,25] In the present study, we report the hitherto unexplored aspect of the effect of 5-FU on collagen synthesis using developing palate as an experimental system to study the drug effect.

Materials and methods

The maintenance and breeding procedures for Golden Syrian hamsters (85 ± 5 g) have been described.[26] On day 11 of gestation, each pregnant hamster was given a single intramuscular injection of either 81 mg/kg 5-FU (Sigma, St Louis, MO; catalog no. F-6627, lot no. 39C-0411) in 1 ml saline, or 1 ml of saline. The latter animals served as controls. This dose and time of drug injection were

This research was supported by grant from the Natural Sciences and Engineering Research Council of Canada.

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chosen because they induce cleft palate in all the embryos without embryolethal effects.[6]

Embryos from both control and 5-FU-treated hamsters were obtained at 4 h intervals between days 11:04 (day 11: 4 h) and 13:00 of gestation. In addition, embryos were also obtained at day 12:02, since in hamster palatal shelf reorientation is completed between days 12:00 and 12:04 of gestation.[14,27,28] The embryonic palatal tissues were dissected under sterile conditions. The dissected palates from each litter ($n = 12 \pm 2$) were pooled and incubated in 4.3 ml Dulbecco's modified Eagle's medium (DMEM) in 60 × 15 mm tissue culture dishes. The incubation medium was supplemented with 0.5 ml of 10% fetal calf serum (FCS), 0.1 ml ascorbic acid (5 mg/100 ml) and 0.1 mg [^{14}C]proline (specific activity 250 μCi/mmol; New England Nuclear, Boston, MA) for 3 h at 37°C in an atmosphere of 5% CO_2–95% air. Following incubation, the samples were washed twice with [^{14}C]proline-free DMEM to remove the excess of radioactive proline from the surface of the tissue. The washed palates were sonicated in 2 ml 0.5 N acetic acid containing 10 mM EDTA at 0–4°C and left at 0–4°C in the refrigerator for 12–18 h protein digestion. The samples were then used for collagen digestion assay, total protein determination and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Collagen digestion assay

The digested samples were dialyzed against 0.5 N acetic acid (0–4°C) with four changes at 6 h intervals. Subsequently, 1 ml of sample was pipetted into an Eppendorf vial for determination of total protein (as described below). The remaining samples were dialyzed further against 0.5 M Tris–HCl buffer, pH 7.4 (0–4°C), as above.

Following dialysis, the radiolabeled samples (0.4 ml) were digested at 37°C for 4 h with 100 μl of 25 units (0.2 mg/ml) bacterial collagenase (Clostridiopeptidase A; EC 3.4.24.3, from *Clostridium histolyticum*, type VII; Sigma, catalog no. C-0773, lot no. 79F6817) in the presence of 0.1 mM *N*-ethylmalemide. Then, to each sample, 10 μl of 10% FCS and 125 μl of a mixture of 10% trichloroacetic acid (TCA) and 1% tannic acid (TA) were added, and left overnight at 0–4°C to precipitate the proteins. Subsequently, the samples were spun in a microcentrifuge at 13 000 r.p.m. for 40 min. The pellets were discarded and 200 μl of the supernatant from each sample was counted in a liquid scintillation counter (Philips, Model PW 4700). The measurement was corrected for the counting efficiency and the disintegrations per minute (d.p.m.) was determined.

Measurement of total protein

The total protein was determined by the Biuret method as described by Gornall and associates.[29] To the acetic acid-dialyzed samples in Eppendorf vials (described above) 250 μl of 10% TCA–1% TA mixture was added. The vials were vortexed and left overnight at 0–4°C. The samples were spun in a microcentrifuge for 30 min and the supernantant discarded. To the precipitate 0.05 N NaOH was added, vortexed and left overnight at 0–4°C for digestion of the protein. Subsequently, 0.1 ml of digest was used for the total protein determination using human albumin and globulin in saline as a protein standard (Sigma, catalog no. 540-10, lot no. 48F-6087).

Each experiment was repeated four or five times and means ± SD were calculated. Both the control and drug-treated data on the rates of collagen synthesis were analyzed by the ANOVA method. The comparison between treated and control groups was evaluated by Student's *t*-test at a significance level of 0.05.

SDS–PAGE

The isotypes of collagen were separated using a method described by Narayanan and Page.[30] Both the drug-treated and control samples, left overnight in 2 ml of 0.5 N acetic acid, as described above, were digested by 200 μg pepsin (100 μg/ml) at 4°C for 24 h. Lathyric rat skin collagen carrier (5 mg) was then added to the samples followed by slow addition of solid NaCl to 1.7 M, to help precipitate the collagen. The mixture was allowed to stand overnight at 4°C and centrifuged (Model L8-70 Ultracentrifuge; Beckman Instruments, Palo Alto, CA) at 27 000 ***g*** for 25 min at 4°C to collect the collagen precipitate. The supernatants were discarded and the pellets dissolved in 0.5 ml of 0.5 N acetic acid containing 10 mM EDTA for a minimum of 24 h at 4°C. Subsequently, the samples were spun in a microcentrifuge at 13 000 r.p.m. for 10 min. The pellets were discarded, supernatant lyophilized and then reconstituted with 100 μl of 0.5 N acetid acid.

SDS–PAGE was performed using 7.5% polyacrylamide in SDS Tris–glycine, pH 8.8.[31] An

aliquot (36 μl) of extracted collagen was dissolved in 'Laemmli' sample buffer (9 μl), boiled for 5 min and loaded into the SDS–PAGE as described by Laemmli.[31] The electrophoresis was performed at 125 V, 75 mA for 15 min. At this stage, the current was switched off and the sample wells filled with 5% v/v 2-mercaptoethanol in sample buffer to separate collagen type I from collagen type III. Mercaptoethanol was allowed to diffuse into the gel for 15 min before the current was again switched on to resume electrophoresis for a further period of 1 h.[32] Subsequently, the gels were dehydrated in dimethylsulfoxide (DMSO), soaked in a solution of 2,5-diphenyloxazol in DMSO to concentrate the bands within the gel. The gels were then dried and each gel was exposed on 8 × 10 in Cronex X-ray (El DuPont de Nemours, Willington, DE) film at −70°C for 15 days. Radiolabeled bands, representing different types of collagen, were identified using ^{14}C-labeled rainbow protein molecular weight marker (specific activity 37 kBq, 1 μCi, Amersham, Buckinghamshire, UK, Batch no. 13, lot no. 4).

Results

Collagen synthesis

In control hamster embryonic palate, between days 11:00 and 12:00 of gestation, i.e. the period when shelves are growing vertically,[14,35] the rate of [^{14}C]proline uptake, indicative of the rate of collagen synthesis, remain unchanged (Figure 1). During the next 4 h, i.e. between days 12:00 and 12:04 of gestation, when the palatal shelves reorient from a vertical to a horizontal plane and fuse,[27,28,33] the rate of collagen synthesis doubles ($p < 0.05$). In the ensuing 4 h the rate decreases. On day 12:08 of gestation, the rate of collagen synthesis is approximately 40% of that seen 4 h earlier ($p < 0.05$).

Following 5-FU treatment, the rate of collagen synthesis in the developing secondary palate of hamster, between days 11:00 and 12:02 of gestation, remains unchanged (Figure 1). During this period, the rate is, however, lower than the controls ($p < 0.01$). It then increases approximately 10-fold to peak at day 12:12 of gestation ($p < 0.01$). In the ensuing 4 h the rate drops 8-fold before showing a 4-fold increase between days 12:16 and 13:00 of gestation, i.e. the period when the 5-FU treated palatal shelves reorient (Figure 1).[15,17]

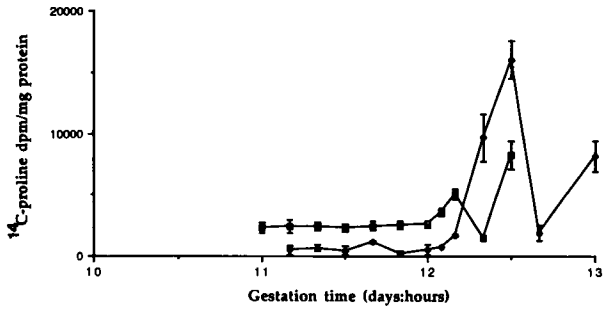

Figure 1. Rate of collagen synthesis in the control (□) and 5-FU-treated (●) developing secondary palate of hamster embryos between days 11 and 13 of gestation.

Measurement of total protein

The control data on the total protein content of the developing secondary palate in hamster are outlined in Figure 2. One may infer from Figure 2 that the amount of total protein in the developing secondary palate doubles between days 11:04 and 12:04 of gestation, i.e. during the period when the shelves have completed vertical growth and reoriented to a horizontal plane and fused ($p < 0.05$). In the ensuing 20 h, i.e. until day 13:00 of gestation, the amount of total protein levelled off.

The results of the measurement of total protein content following 5-FU treatment are summarized in Figure 2. In general, between days 11:04 and 13:00 of gestation, the total protein content in 5-FU-treated palates was approximately 50–75% lower than in control palates ($p < 0.05$). Also, unlike controls, the amount of total protein in 5-FU-treated palates is unchanged between days 11:04 and 13:00 of gestation, i.e. the period during which the cleft palate was forming.

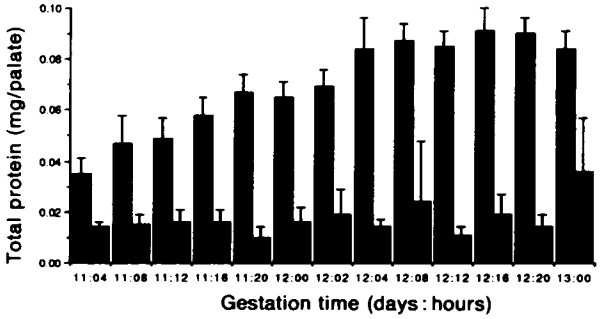

Figure 2. Total protein content in the control (■) and 5-FU-treated (■) developing secondary palate of hamster embryos between days 11:04 and 13:00 of gestation.

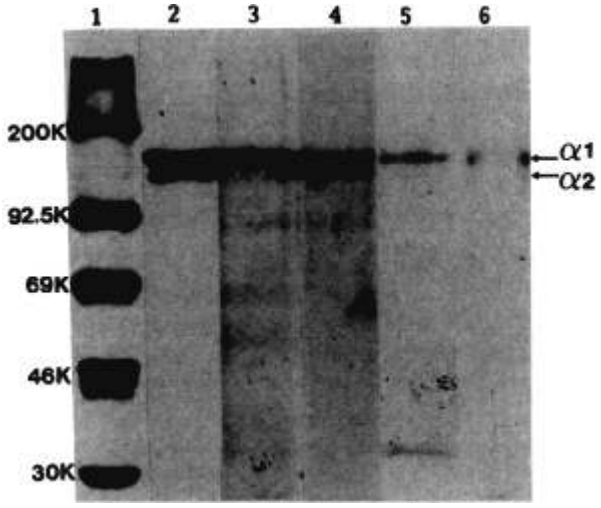

Figure 3. SDS–PAGE showing type I collagen in the control and 5-FU-treated developing secondary palate of hamster embryos on days 12:02 and 12:16 of gestation. Track 1. Molecular weight marker (14 300–200 000). Track 2. Standard type I collagen showing α1 and α2 chains. Track 3. Control secondary palate of hamster embryo (12:02). Track 4. 5-FU-treated secondary palate of hamster embryo (12:02). Track 5. Control secondary palate of hamster embryo (12:16). Track 6. 5-FU-treated secondary palate of hamster embryo (12:16).

SDS–PAGE

The results of SDS–PAGE to separate different collagen isotypes in the developing palate of hamster embryos are presented in Figure 3. Collagen types were determined in both the control and drug-treated animals at the time of shelf reorientation. In control hamster embryos only type I collagen was detected by SDS–PAGE. Similarly after 5-FU administration, only type I collagen was identifiable by SDS–PAGE at the time of delayed reorientation of the palatal shelf (Figure 3). In both controls and drug-treated palates, a delayed reduction was performed using 2-mercaptoethanol to examine if type I and type III collagen were bound. No separate bands were observed by SDS–PAGE suggesting that either type III collagen is present in such a small quantity that it was not possible to detect it by SDS–PAGE in the present study or that it does not exist in the developing secondary palate of hamster.

Discussion

In the present study, a changing profile of the rate of collagen synthesis in the normally developing secondary palate of hamster embryos was observed. Specifically, at the time of shelf reorientation in hamster, the rate of collagen synthesis showed a 2-fold increase indicating that at least some radiolabeled proline was utilized for the synthesis of new collagen. Earlier studies[18,20] observed a 2-fold increase in the rate of collagen synthesis in the normally developing palatal shelves of mice *before* shelf reorientation, or doubling the amount of collagen *at the time of palate closure* in rats,[19] or an 82% increase in the rate of synthesis in the vertical shelf, *prior to* shelf reorientation.[21] One of the drawbacks of these earlier studies on mice and rats was that the collagen synthesis was analyzed at 12–24 h intervals. In such experimental circumstances, subtle changes in the rates of collagen synthesis *during* the shelf reorientation (which in mammals occurs very rapidly in 2–3 h[33–35]) were not localized. Nevertheless these observations, along with the data from the present study, suggest that, at least during mammalian palatogenesis, a rise in the synthesis of new collagen may be related to the reorientation of the palatal shelves.

The issue was further evaluated by using 5-FU because, in hamster, 5-FU delays the reorientation of shelves to induce a cleft palate.[15,16] It was thought that, if 5-FU affected collagen synthesis and if the synthesis of new collagen was critical for shelf reorientation, then the synthesis of new collagen would be delayed to correspond with the 5-FU-induced delayed reorientation of shelf. Observations of the present study showed that between days 12:00 and 12:04 of gestation, which is the time of shelf reorientation, collagen synthesis in 5-FU-treated palate was depressed and the shelf reorientation was delayed. Subsequent rise in collagen synthesis on day 12:12 of gestation occurred only a few hours prior to shelf reorientation. In a series of earlier studies, it was shown that within 6 h of drug administration, 5-FU injured a significant proportion of cells in the vertically developing palate of hamster[16] and subsequently delayed the expansion of the shelf volume.[17] The reorientation of shelves occurred only after the resolution of cellular damage on day 12:06 and subsequent restoration of shelf volume on day 12:12 of gestation. The increasing collagen synthesis in the treated palates between days 12:04 and 12:12 of gestation thus correlates well with the restoration of shelf volume and may be one of the important events of the recovery phase. In a recent study, it was observed that, following 5-FU administration, initial alteration in the synthesis of GAG was also quickly restored prior to the time of normal shelf reorientation in hamster.[25] Hence,

it is likely that altered cytodifferentiation due to 5-FU-induced cell injury[16] would in turn affect GAG[25] and collagen synthesis (present study), and consequently the expansion of the shelf volume.[17] Thereafter, in 5-FU-treated palates, an increase in collagen synthesis between days 12:16 and 13:00 of gestation corresponded well with shelf reorientation. With the exception of our previous study,[36] there are no reports in the literature on the effects of FU on collagen synthesis in any developing system. Hence, the results of the present study are difficult to compare. On the basis of the data of the present study, however, one may suggest that (i) new synthesis of collagen may cause the shelf to reorient and (ii) a certain minimum accumulation of collagen in the developing palate may be necessary to achieve the critical shelf volume[17] before reorientation of the palatal shelf could occur.

Since proline is an amino acid ubiquitous to most proteins[37] and since during collagen synthesis only a limited amount of proline is incorporated into collagen, the question of whether the profile of total protein accumulation in both the control and 5-FU-treated developing palate of hamster would follow that of collagenous protein was examined. The results indicated that although in the control palate the protein accumulation increased during the shelf reorientation, it remained suppressed following 5-FU treatment. Since, during the reorientation phase, collagen synthesis continues, the reduction in total protein would reflect the effect of 5-FU also on non-collagenous protein synthesis. This deduction would be consistent with the previously noted effect of 5-FU on the reduction of protein synthesis in various individual organs and tissues.[3,11–13,38]

Although collagen has been implicated to play a role in different biological processes, its precise role during various normal and abnormal circumstances has begun to emerge only during recent years. For example, it has been indicated that both types I and III are involved in the development of the branching pattern of the salivary glands[39–40] and during wound healing.[41–42] In these circumstances, type I collagen provides a lattice network to facilitate proliferation and migration of cells.[38,42–43] During the development of a limb, a structure which, like the secondary palate, grows outward from the body surface, increasing synthesis of type I collagen has been related to cell orientation and arrangement.[44–45] As observed in the present study, as well as noted by previous investigators,[18–22] the developing secondary palate of mammals shows synthesis of type I collagen during its vertical growth and reorientation stages. The migratory behavior of mouse palate mesenchymal cells cultured on a substrate of collagen gel has also been observed.[46] Hence, it is reasonable to suggest that during the formation of the mammalian secondary palate, type I collagen may contribute both to the volume of the shelf through various stages of palatogenesis as well as to the rigidity of the substratum on which cells may perform their various biological functions. Inhibition of collagen synthesis during abnormal development is only a step in the cascade of events of the 5-FU-induced effect on protein synthesis.

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(Received 3 September 1993; accepted 9 September 1993)