Collagen synthesis in fibroblasts from human colon: regulatory aspects and differences with skin fibroblasts

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Abstract

The purpose of this study was to examine regulation of collagen synthesis in human colon fibroblasts and compare the results from colon fibroblasts with those obtained in fibroblasts from human skin. The effects of interleukin-1 β , tumour necrosis factor α , interferon gamma, transforming growth factor- β , dexamethasone, and the calcium ionophore A23187 were investigated. All compounds were tested both in the absence and in the presence of fetal calf serum in the culture medium. The process of collagen synthesis in fibroblasts from colon and skin appears to be affected differently by these regulatory compounds. The most pronounced differences were that the relative collagen synthesis increased in dermal fibroblasts and decreased in colon fibroblasts upon addition of serum. In the presence of serum, interleukin-1 β inhibited collagen synthesis in skin fibroblasts but not in colon fibroblasts. Dexamethasone suppressed the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts. Transforming growth factor- β stimulated the collagen synthesis in dermal fibroblasts in the presence of serum, but inhibited the process in colon fibroblasts. Because fibroblasts are the primary sources of collagen needed during wound repair, these results may offer (part of) the explanation why wounds in skin and intestine appear to behave differently under certain conditions.

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Wound healing is defined as a highly regulated sequence of various cellular events leading to reconstitution of tissue integrity after injury.¹ Fibroblasts play an important role in this sequence by producing extracellular matrix components such as the different types of collagen and fibronectin.²⁻⁴ Collagen is particularly important for the development of wound strength. In

skin wounds, the increase in hydroxyproline parallels the increase in tensile strength.⁵ Also, a direct relationship is observed between collagen diameter and tensile strength.⁶ Collagen metabolism is closely controlled during the process of wound repair. Regulation is probably achieved by the interaction of fibroblasts with the surrounding extracellular matrix as well as by cytokines and growth factors known to regulate specifically collagen gene expression and collagen metabolism.⁷⁻⁹

Most studies on wound healing have used skin, mainly because of the easy accessibility of this tissue. Although one would certainly like to think that all wounds heal by a single common mechanism, various authors¹⁰⁻¹² have expressed caution in extrapolating the results from research on skin repair to the healing of other soft tissues such as intestine. Anastomotic repair in the intestine has been a topic of study in our laboratory for some time.¹³ We have indeed found indications that intestinal wounds and skin wounds react differently to in vivo administration of various drugs. For instance, administration of methylprednisolone does not affect strength or collagen content of intestinal anastomoses while impairing healing in skin.¹⁴ Also, administration of D-penicillamine, a lathyrogen which inhibits collagen cross linking, leaves anastomotic strength and collagen solubility unaffected while it lowers strength and increases collagen solubility in skin wounds.¹⁵ Although various explanations for these phenomena are possible, the question arises if production and metabolism of collagen in wounds of intestine and skin are under different control.

Because fibroblasts are the primary collagen producing cells in healing wounds, we decided to study the regulation of the collagen production rate in fibroblasts from human colon. Although recent work describes enhanced collagen gene expression in 'fibroblast like' cells in experimental colonic anastomoses,¹⁶ no quantitative data are available as yet regarding collagen synthesis in colon fibroblasts. We have compared the results with those obtained with fibroblasts from human skin. The data show that synthesis of collagenous protein in cells from both tissues is affected differently by various regulatory compounds.

Methods

MATERIALS

All supplies for cell culture were purchased from Life Technologies (Breda, The Netherlands). Transforming growth factor- β from bovine bone

TABLE 1 Effect of serum on collagen and non-collagenous protein synthesis

	HSF		HCF	
	10% serum	0% serum	10% serum	0% serum
Dpm Collagenase digestible protein/well	27 299 (7299)	7194 (2979)	56 730 (5904)	20 842 (589)
Dpm Non-collagenous protein/well	164 301 (34 204)	114 442 (52 967)	464 064 (40 956)	117 774 (33 346)
% RCS	3.09 (0.49)	1.30 (0.69)	2.22 (0.06)	3.27 (0.44)

Synthesis was measured in both cell lines cultured in the presence and absence of fetal calf serum. Results represent average values (SD) from five separate experiments.
HSF=human skin fibroblasts; HCF=human colon fibroblasts.

was a gift from Dr G Ksander (Celtrix Labs, Palo Alto, USA). Interferon gamma was obtained from Boehringer (Ingelheim, Germany). Both

TABLE II Effect of IL-1 β on collagen and non-collagenous protein synthesis

	IL-1 β U/ml	HSF		HCF	
		10% serum	0% serum	10% serum	0% serum
Collagenase digestible protein/well	0	100 (14)	100 (6)	100 (10)	100 (6)
	1	77 (14)	64 (10)*	108 (19)	103 (14)
	50	69 (2)*	50 (9)*	104 (8)	49 (5)*
Non-collagenous protein/well	0	100 (13)	100 (6)	100 (9)	100 (12)
	1	98 (12)	98 (11)	106 (12)	112 (5)
	50	98 (11)	83 (11)	126 (6)*	90 (10)
%RCS	0	100 (11)	100 (3)	100 (3)	100 (5)
	1	78 (4)*	67 (4)*	102 (8)	92 (12)
	50	67 (4)*	62 (4)*	83 (3)*	56 (12)*

Both colon fibroblasts (HCF) and skin fibroblasts (HSF) were grown in 10% serum or serum free culture medium with different concentrations of interleukin-1 β . Results are expressed as percentile values with regard to the control cultures without interleukin-1 β . Data represent average values (SD) of four cultures. Differences between control and cytokine treated cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

Figure 1: Effect of tumour necrosis factor- α (TNF- α) on collagen and non-collagenous protein synthesis. Visually confluent fibroblasts from both cell lines were incubated for 48 hours with or without tumour necrosis factor- α , both in the presence or absence of fetal calf serum. Results are expressed as percentage of control without added tumour necrosis factor- α . Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (SD) of four cultures is given. Differences between control and cytokine treated cell cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$. Abbreviations see Table I.

human recombinant interleukin-1 β and tumour necrosis factor α were a gift of Professor Dr J W M van der Meer (University Hospital Nijmegen). L-[2,3- 3 H]proline (1,63 Tbq/mmol) was purchased from Amersham International, England. Collagenase (type VII), calcium ionophore (A23187) and dexamethasone were obtained from Sigma (St Louis, USA). All other reagents were of analytical grade (Merck, Darmstadt, Germany).

CELL CULTURE

Normal human colon fibroblasts were obtained from the American Type Culture Collection (CRL-1459). Human skin fibroblasts were obtained from explants of skin biopsies of a healthy adult. Both the human skin fibroblasts and human colon fibroblasts were grown in Dulbecco's Modified Eagles Medium supplemented with antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin) and 10% fetal calf serum at 37°C in a 5% CO $_2$, 95% air humidified atmosphere. Cells were used between the third and tenth passage.

ASSAY OF FIBROBLASTS COLLAGEN PRODUCTION

Collagen production by steady state, visually confluent fibroblasts was assessed over a 24 hour period by [3 H]proline incorporation into collagenous protein.

Freshly trypsinised fibroblasts were plated in six well plates at a density of approximately 1.5×10^5 cells/well in 2 ml Dulbecco's Modified Eagles Medium plus 10% fetal calf serum. Three days after plating the medium was removed and replaced by the same medium or with Dulbecco's Modified Eagles Medium without serum. In the latter case the wells were first washed twice with phosphate buffered saline. Twenty four hours later the medium was replaced by the same medium plus ascorbic acid (50 μ g/ml), β -aminopropionitrile (50 μ g/ml) and 2 μ Ci/ml [2,3- 3 H]proline for the final 24 hours of culture. Interleukin-1 β , tumour necrosis factor α , interferon gamma, transforming growth factor- β , dexamethasone or calcium-ionophore A23187 were added during the labelling period. Interleukin-1 β and tumour necrosis factor α were also added during the 24 hour culture period before the labelling period. If the calcium ionophore was given, 1.5 mM CaCl $_2$ was simultaneously added to the culture medium.

After the labelling period the cells and medium were scraped from the wells and the wells were washed twice with 1 ml 50 mM Tris-HCl pH 7.6 containing 25 mM ethylenediaminetetraacetic acid, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulphonylfluoride and 1 mM proline. The wash solution was added to the suspension which contained cells and medium. The final suspension was freeze/thawed three times and the proteins were precipitated with trichloroacetic acid (final concentration 10%). The radioactive protein was separated from free [3 H]proline by repeated (3 $\times$) washes with 5% trichloroacetic acid containing 1 mM proline at 4°C.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralised by the addition of

0.3 ml 1 M HEPES and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM CaCl₂ and 0.1 ml collagenase (chromatographically purified on a G200 gel filtration column) were added to a 0.5 ml aliquot of the solubilised sample and the mixture was incubated for five hours at 37°C. The digestion was terminated by the addition of trichloroacetic acid and tannic acid up to final concentrations of 0.6 M and 3 mM, respectively. After centrifugation (10 minutes, 14.500 g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyser. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase

yielded the collagen specific incorporation, which will be referred to as collagenase digestible protein. Subtraction of the radioactivity in the collagenase digestible protein fraction from that in total protein yields the incorporation into non-collagenous protein. Incorporation into collagenase digestible protein and non-collagenous protein is quantified per well.

The relative collagen synthesis was calculated with the formula¹⁷ that takes into account the enrichment of proline in collagen compared with other proteins:

$$\% \text{ relative collagen synthesis} = \frac{\text{collagenase digestible protein}}{(\text{non-collagenous protein} \times 5.4) + \text{collagenase digestible protein}} \times 100\%$$

Results

Collagen synthesis in the two fibroblast strains was always measured the presence and in the absence of 10% serum. In the presence of serum, human colon fibroblasts produce more collagen and non-collagenous protein than human skin fibroblasts (Table I). This difference is maintained if the incorporation of label into the fractions is expressed per cell (results not shown). If serum is omitted, synthetic capacity decreases. The absolute collagen synthesis is lowered by 63 and 74% in human colon fibroblasts and human skin fibroblasts, respectively. The synthesis of non-collagenous protein, however, is affected far more strongly in human colon fibroblasts. As a result, serum free conditions cause the relative collagen synthesis to be more than halved in human skin fibroblasts and to be increased by nearly 50% in human colon fibroblasts.

Cytokines are potential regulators of protein synthesis. In human colon fibroblasts cultured with serum the absolute collagen synthesis is not affected by interleukin-1 β (Table II). As the synthesis of non-collagenous protein increases with the higher concentration of interleukin-1 β used, the relative collagen synthesis is lowered. In the absence of serum, both absolute and relative collagen synthesis are inhibited by 50 U/ml interleukin-1 β . The collagen synthesis in skin fibroblasts appears far more susceptible to the cytokine; the lower concentration suppresses synthesis independent of the presence of serum. The bulk of non-collagenous protein synthesis remains unaffected by the addition of interleukin-1 β .

Tumour necrosis factor α , at a concentration of 2 ng/ml, does not affect the synthesis of non-collagenous protein in either of the fibroblast strains (Fig 1). If the skin fibroblasts are cultured without serum, no effect on collagen synthesis is observed. In the presence of serum, the relative collagen synthesis is reduced by 14%. The potentially inhibitory effect of tumour necrosis factor α is more explicit in the human colon fibroblasts under both culture conditions. For instance, the absolute collagen synthesis is reduced by 50% if cells are grown without added serum. At lower concentrations, 0.02 and 0.2 ng/ml, tumour necrosis factor α induces no alterations in synthetic activity (results not shown).

Both cell lines react essentially similar to the addition of interferon gamma to the culture

Figure 2: Effect of interferon gamma on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines were incubated for 24 hours with or without interferon gamma, both in the presence or absence of fetal calf serum. Results are expressed as percentage of control without added interferon gamma. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (SD) of four cultures is given. Differences between control and cytokine treated cell cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

medium. Again, total protein synthesis remains unaffected, while collagen synthesis is lowered (Fig 2). In human skin fibroblasts however the decrease is more apparent in the presence of serum while in human colon fibroblasts significantly reduced collagenase digestible protein and %relative collagen synthesis values are only observed after culture without serum.

The synthetic activity in human colon fibroblasts is hardly affected by the glucocorticoid dexamethasone (Fig 3): in the presence of serum neither the collagen synthesis nor the synthesis of non-collagenous protein changes after addition of the drug. Both activities appear to be stimulated in serum free cultures, at least at the higher concentrations of dexamethasone. As a result, the relative collagen synthesis remains

unaffected. Human skin fibroblasts react differently; in the presence of serum the synthesis of non-collagenous protein is doubled by dexamethasone while the absolute collagen synthesis is not influenced. Thus, the relative collagen synthesis decreases significantly, from concentrations of 10^{-9} M dexamethasone upwards.

Another way to regulate collagen synthesis in colon fibroblasts is the addition of the calcium ionophore A23187. While no effects are observed in the presence of serum (results not shown), culturing cells in the presence of $0.6 \mu\text{M}$ A23187 suppresses the collagen synthesis by more than 75% (Fig 4). Although human skin fibroblasts are also found to be susceptible to the presence of the ionophore the picture seems somewhat different: the significant decrease in the relative collagen synthesis appears more a result from an increase in synthesis of non-collagenous protein than from a lowered absolute collagen synthesis.

Growth factors, and especially transforming growth factor- β , are thought to stimulate fibroblast collagen synthesis. This is indeed the case in human skin fibroblasts, regardless of culture conditions (Fig 5). In both cases, the absolute and relative collagen synthesis are increased, although the latter less so in 10% serum since here the synthesis of non-collagenous protein is also significantly enhanced. Human colon fibroblasts react just the opposite, at least in the presence of serum. Both collagen and non-collagenous protein synthesis are suppressed significantly. In the absence of serum, collagen synthesis in human colon fibroblasts is enhanced by transforming growth factor- β , particularly at a concentration of 5 ng/ml. The synthesis of other proteins, however, is stimulated to the same extent and thus the relative collagen synthesis remains unchanged.

Finally, we investigated if transforming growth factor- β could negate the inhibitory effects of interferon gamma and A23187 on collagen synthesis in colon fibroblasts cultured under serum free conditions. As shown before, both interferon gamma and A23187 significantly suppress collagen synthesis, absolute and relative, while transforming growth factor- β stimulates absolute collagen synthesis. Transforming growth factor- β is able to overcome the negative effects of interferon gamma (Table III): if added simultaneously, values for the parameters observed are similar to those observed in the presence of transforming growth factor- β alone. In contrast, transforming growth factor- β cannot restore the inhibition induced by A23187. Culture in the presence of both compounds results in a massive suppression of collagen synthesis, similar to that induced by A23187 alone.

Discussion

Collagen synthesis is an essential and universal feature of wound repair. After construction of an anastomosis in the intestine the synthetic capacity is strongly, and specifically raised in the wound area.^{18,19} Because collagen synthesis is thought to be crucial to the development of anastomotic strength, the study of its regulation is of great potential interest. Leakage of colonic

Figure 3: Effect of dexamethasone on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines were incubated for 24 hours with or without dexamethasone, both in the presence or absence of fetal calf serum. Results are expressed as percentage of control without added dexamethasone: black bars represent controls, striped bars 10^{-9} M, dotted bars 10^{-7} M and open bars 10^{-5} M dexamethasone. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (SD) of four cultures is given. Differences between control and dexamethasone treated cell cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

anastomoses is a phenomenon that occurs quite frequently²⁰ and insight into regulation of the processes which are essential in the repair sequence could contribute to the development of measures to avoid this complication. The cells most likely to be responsible for collagen synthesis are the fibroblasts which migrate into the wound and display great proliferative activity. Thus, we investigated collagen synthesis in an established line from human colon fibroblasts²¹ and compared the results with those obtained with fibroblasts from human skin. In the literature on fibroblast collagen synthesis discrepancies between studies are often explained by supposedly different culture conditions, particularly with respect to the presence of serum.²² In order to discover if such variations in culture conditions indeed change the effects of the

regulatory compounds studied, synthetic activity was measured both in the presence and absence of serum.

It has been shown that addition of serum to fibroblast cultures stimulates collagen synthesis,²³ probably at the transcriptional level.²⁴ Although colon fibroblasts behave similarly in this respect they seem to be unique in the sense that the synthesis of non-collagenous protein is stimulated to a greater degree, resulting in a lower relative collagen synthesis under serum-supplemented conditions.

Interleukin-1 β inhibits collagen synthesis in colon fibroblasts cultured in the absence of serum. We found similar results for skin fibroblasts, which observation is in agreement with those reported by others.^{22, 25} The fact that such inhibition was not seen in the presence of serum confirms a similar observation by Duncan and Berman,²⁶ although our skin fibroblasts are also responsive under these conditions and display a suppressed collagen synthesis.

Tumour necrosis factor α inhibits the collagen synthesis in colon fibroblasts. A similar effect with comparable tumour necrosis factor concentrations has been reported for skin fibroblasts,^{27, 28} while our own results show that only the relative collagen synthesis decreases in skin fibroblasts cultured in the presence of serum. These data contradict those of others²⁶ who show an increased collagen synthesis in dermal fibroblasts cultured without serum.

Studies on interferon gamma show that this compound universally inhibits collagen synthesis in a variety of target cells – for example, fibroblasts^{27, 29} and fetal bone cultures.³⁰ In this respect, colon fibroblasts are no exception although significant inhibition is only measured under serum free conditions.

Neither the absolute nor the relative collagen synthesis in colon fibroblasts are significantly affected by dexamethasone. Most studies with skin fibroblasts indicate that glucocorticoids reduce collagen synthesis³¹: decreased concentrations of mRNA suggest pretranslational regulation.³² In the present study, a reduction in the absolute collagen synthesis is only apparent, and not significantly so, if dermal fibroblasts are grown without serum. The decreased relative collagen synthesis is, certainly in serum supplemented cultures, the result of a stimulation in the synthesis of non-collagenous protein. In animal studies we have found that colonic wound healing is unaffected by administration of corticosteroids at doses which impair dermal wound healing.¹⁴ Therefore, it is interesting to observe that collagen synthesis in fibroblasts from colon seems refractory to corticosteroids. Possibly, a difference in regulation of this fibroblast function may (partly) explain the divergent healing patterns in both tissues.

Growth factors, and especially transforming growth factor- β , are known to stimulate collagen synthesis in fibroblasts from a variety of tissues.³³⁻³⁵ Our results with dermal fibroblasts confirm earlier results reported for cells from this tissue. In contrast, synthetic activity in human colon fibroblasts is significantly inhibited in the presence of serum. While there has been a report on negative effects of transforming growth

Figure 4: Effect of calcium ionophore A23187 on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines were incubated for 24 hours with or without A23187, in absence of fetal calf serum. Results are expressed as percentage of control without added A23187. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (SD) of four cultures is given. Differences between control and ionophore treated cell cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

Figure 5: Effect of transforming growth factor-β on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines were incubated for 24 hours with or without transforming growth factor-β, both in the presence or absence of fetal calf serum. Results are expressed as percentage of control without added transforming growth factor-β. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (SD) of four cultures is given. Differences between control and growth factor treated cell cultures are tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

factor-β on collagen synthesis in hepatocytes,³⁶ this is the first evidence that transforming growth factor-β may inhibit collagen synthesis in fibroblasts. Fukamizu and Grinnell³⁷ described decreased collagen synthesis in long term fibroblast cultures, possibly as the result of lowered

fibroblast activity concomitant with spatial reorganisation of the extracellular matrix. In the absence of serum, absolute collagen synthesis in colon fibroblasts is indeed stimulated by transforming growth factor-β in a concentration of 5 ng/ml. At this concentration, transforming growth factor-β can overcome the inhibition induced by interferon gamma under these conditions, suggesting that both cytokines affect collagen synthesis through the same regulatory pathway. Simultaneous exposure of either skin fibroblasts³⁸ or gingival fibroblasts³⁹ to both interferon gamma and transforming growth factor-β did reverse the stimulation in collagen production observed with transforming growth factor-β alone, suggesting the presence of independent mechanisms.

Increasing the intracellular calcium concentration by addition of the ionophore A23187 together with extra calcium to the culture medium strongly suppresses collagen synthesis in colon fibroblasts. If the ionophore is added together with transforming growth factor-β, collagen synthesis remains inhibited to the same extent. Thus it seems that the regulatory action of transforming growth factor-β is dependent upon a low intracellular calcium level.

Next to investigating the behaviour of collagen synthesis in colon fibroblasts under addition of various regulatory compounds, the aim of this study was to establish if fibroblasts from colon and skin behave differently in this respect. This indeed appears to be the case. The relative collagen synthesis increases in dermal fibroblasts and decreases in colon fibroblasts upon the addition of serum. In the presence of serum interleukin-1β inhibits collagen synthesis in skin fibroblasts but not in colon fibroblasts. Dexamethasone suppresses the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts. Transforming growth factor-β stimulates the collagen synthesis in dermal fibroblasts cultured in the presence of serum but inhibits the process in colon fibroblasts.

The other differences observed are less explicit and merely a matter of degree and fall within the variations observed by us (results not shown) and others²⁶ for different cell lines from the same tissue. Those mentioned above, however, and most particularly the discrepancy measured in the presence of transforming growth factor-β, suggest that repair activities of fibroblasts in colon and skin may be under different control. Wound fibroblasts obtained from implanted sponges differ from normal dermal fibroblasts with regard to their capacity to synthesise collagen and remodel collagen lattices.⁴⁰ Thus, the wound environment may alter fibroblast phenotype. Our results indicate that fibroblasts from skin and colon may exhibit divergent reactions to cytokines or growth factors produced during the inflammatory part of the healing sequence and therefore may cause wounds in skin and intestine to behave differently under certain conditions.

TABLE III Collagen synthesis in human colon fibroblasts under serum free conditions

	CDP/well	NCP/well	%RCS
Control	100 (13)	100 (13)	100 (11)
+ Transforming growth factor-β (10 ng/ml)	140 (10)*	146 (4)*	95 (5)
+ Interferon gamma (1000 u/ml)	61 (5)*	82 (6)	76 (9)*
+ Interferon gamma + transforming growth factor-β	152 (8)*	153 (9)*	99 (5)
+ A23187 (0.6 μM)	14 (2)*	71 (7)*	20 (1)*
+ A23187 + transforming growth factor-β	16 (2)*	79 (3)*	21 (2)*

Cells were grown in serum free medium. Results are expressed as percentile values with regard to control cultures. Data represent average values (SD) of four cultures. Differences between control and other cultures were tested for significance using a two sided Wilcoxon's test: * $p \leq 0.05$.

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