Hydrocortisone modulates the expression of c-ets-1 and 72 kDa type IV collagenase in chicken dermis during early feather morphogenesis

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ABSTRACT At the onset of chicken feather morphogenesis, dermal cells migrate along bundles of collagen fibers to colonize areas where bud outgrowth takes place. Chicken embryos treated with hydrocortisone during the critical phase of dermal rearrangement show featherless skin areas in which the dermis exhibits an increase of interstitial collagen. We had previously demonstrated that c-ets-1 is a nuclear transcription factor expressed in the dermis at the beginning of feather morphogenesis. Here we study, by in situ mRNA hybridization, the expression of c-ets-1 in the dermis of chicken embryos treated with hydrocortisone. We found that, among the two distinct products (p54 and p68) encoded by the chicken c-ets-1, the expression of the p68 product increased while expression of p54 decreased after hydrocortisone treatment. Since Ets-1 regulates matrix-metalloproteinases genes, we analyzed the expression of the 72 kDa type IV collagenase in both normal and hydrocortisone-treated embryos. We demonstrated that 72 kDa type IV collagenase mRNA expression decreased in the dermis after hydrocortisone treatment and that its expression correlated with that of p54-c-ets-1. Taken together, these results indicate that hydrocortisone modulates c-ets-1 expression. In addition, they raise the interesting possibility that c-ets-1 might be involved in an altered pattern of feather development mediated by the accumulation of collagen due to a decrease in collagenase activities.

KEY WORDS: dermis, feather morphogenesis, c-ets-1, matrix-metalloproteinase, c-jun, hydrocortisone

Introduction

In chicken, feather morphogenesis results from interactions between epidermis and dermis. These interactions lead to specific spatio-temporal patterns of cellular division, migration and differentiation. After a first wave of mesenchymal accumulation underneath the epidermis, the formation of epidermal placodes triggers a spatial rearrangement of dermal cells leading to the aggregation of these cells under each epidermal placode (Sengel, 1957, 1970, 1990; Sengel and Rusauèn, 1968; Wessels, 1965; Chuong and Edelman, 1985; Desbiens et al., 1991). At least two distinct extracellular matrix components are involved during this process: fibronectin, which allows cellular adhesion, and interstitial collagen (Mauger et al., 1982), which is arranged in a fibrillar network linking the centers of rising feather rudiments. It has been proposed that dermal cells migrate along these bundles to colonize the sites of aggregation beneath epidermal placodes (Dhouailly, 1984). It has also been reported that hydrocortisone, a glucocorticoid hormone synthesized by adrenal glands, is able to modify feather formation when administered at the onset of dermal cell migration (Züst, 1971). Indeed, treatment with hydrocortisone leads to a depletion of fibronectin and a uniform accumulation of interstitial collagen in the dermal extracellular matrix causing disruption of the collagen fiber network and the subsequent loss of dermal cell migration towards aggregating centers (Demarchez et al., 1984). In addition, the normal proliferation pattern was conspicuously modified in the two skin compartments (Desbiens et al., 1992).

Proto-oncogenes control both cell proliferation and differentiation. During chicken skin development, the expression pattern of three proto-oncogenes encoding nuclear transcription factors exhibit peculiar features: c-myc and c-myb are expressed in epidermal and dermal cells when a high rate of cellular proliferation is detected. In contrast c-ets-1 is solely detected in undifferentiated proliferating dermis which undergoes spatial reorganization, and later in endothelial cells which invade newly established feather buds (Desbiens et al., 1991). Since c-ets-1 is expressed in chicken dermis at the glucocorticoid-sensitive onset of feather morphogenesis (Desbiens et al., 1991; Quèva et al., 1993), we
decided to study the effect of glucocorticoids on c-ets-1 expression. Ets-1 belongs to a growing family of transcription factors which share the ETS domain, a 85 amino acid strongly conserved in the DNA-binding domain (MacLeod et al., 1992). C-ets-1 encodes, by alternate promoter usage, two nuclear proteins, p68 and p54 (Crepieux et al., 1993). The two transcription factors contain the same DNA-binding but differ in their transactivation domains: the p68 contains two transactivation domains, one of which is common to the p54 isoform (Schneikert et al., 1992). The p54 and p68 proteins are expressed in a wide population of mesodermal cells of the chicken embryo, particularly in dermis (Quéva et al., 1993).

The Ets-family of transcription factors regulate cellular genes such as collagenase 1 and stromelysin 1 (Gutman and Wasylyk, 1990; Buttice and Kurkinen 1993; Buttice et al., 1996). The two genes belong to a family of matrix-metalloproteases (MMPs) that degrade several components of the extracellular matrix (ECM) such as collagen, fibronectin, laminin, tenascin and proteoglycans. Active turnover of the extracellular matrix molecules occurs in several steps of growth, development and morphogenesis, suggesting that the MMPs play a major role in these processes (Alexander and Werb, 1989; Birkedal-Hansen, 1995). We and others have recently demonstrated that one of the MMPs gene, the chicken 72 kDa type IV collagenase, is expressed in early development (Yang et al., 1996) and that its expression is particularly abundant in the dermis and feather buds of chicken at later stages of development (N.T. and G.B. unpublished data). To understand the effect of hydrocortisone on feather morphogenesis, we first investigated the expression pattern of c-ets-1 during feather formation and compared it with the 72 kDa type IV collagenase. We show that hydrocortisone treatment of chicken embryos results in an increase of p68c-ets-1 expression, and a decrease in p54 and 72 kDa type IV collagenase expression. Modulations of c-ets-1 and 72 kDa type IV collagenase expression take place in the dermis and probably accompany concomitant disruption of the collagen fiber network.

Fig. 1. Expression of Ets1 and Jun in dermis of transverse sections of chicken embryos. Chicken embryos were treated with PBS as control, or with 0.1 mg of hydrocortisone at E6.5 and fixed 8 h or 24 h (not shown) after injection. Sections from dorsal dermis were hybridized with a sense (not shown) and antisense mRNA common probes for both c-ets-1 transcripts encoding p68c-ets-1 and p54c-ets-1 and for c-jun. As previously described (Desbiens et al., 1991), c-ets-1 transcripts are abundant in the dermis at E6.5 (a and d) and even in the following 8 h (b and e). The c-ets-1 signal strongly decreases in the dermis of hydrocortisone-treated embryos (arrowhead) (c and f). C-jun is weakly expressed in chicken dermis at E6.5 (g and j) or at E6.5+8 h (h and k). C-jun expression is higher in motoneurons (mt). In hydrocortisone-treated embryos, c-jun is more expressed in the neural tube and in the dermis (compare h and i or k and l). nt, neural tube; mt, motoneurons. Note that panels a, b, c, g, h and i represent an enlargement of the dermis. Bars: a, b, c, g, h, i, 75 μm; d, e, f, j, k, l, 190 μm.
Fig. 2. Differential expression of p54 and p68 mRNA in the dermis of chicken embryos. We used sense (not shown) and antisense mRNA probes for p54 and p68 c-ets-1 transcripts. The signal detected with the p54 probe in the dermis at E6.5 (a, d and g) and 8 h later (b, e and h) was reduced in hydrocortisone-treated embryos (arrowhead) (c, f and i). In contrast, the signal detected with the p68 probe in dermis at E6.5 (j, m and p) and 8 h later (k, n and q) was increased in the hydrocortisone-treated embryos (arrowhead) (l, o and r). nt, neural tube. Bars: g, h, i, p, q, r, 35 μm; d, e, f, m, n, o, 75 μm; a, b, c, j, k, l, 190 μm.

Results

Hydrocortisone modulates c-ets-1 mRNA expression in chicken dermis

Transverse sections of chicken embryos at E6.5 (Fig. 1a and d) and control embryos treated with PBS (Fig. 1b and e) or hydrocortisone (Fig. 1c and f) for 8 h were hybridized with a 35S c-ets-1 antisense probe recognizing both p54 c-ets-1 and p68 c-ets-1 encoding mRNA. As shown in Figure 1a, d, b and e, c-ets-1 was expressed in normal dermis confirming previous results (Desbiens et al., 1991; Queva et al., 1993). In hydrocortisone-treated chicken embryos, c-ets-1 expression clearly decreased in dermis (Fig. 1c and f). Weak expression persisted in the neural tube and vessels of the hypoderm and those lining the vertebral arches. Thus, in different tissues expressing c-ets-1, hydrocortisone treatment resulted in a decrease of c-ets-1 mRNA.

We were concerned that the decrease of c-ets-1 expression could be a result of a general repression effect due to the hydrocor-
tisone treatment. Thus, in control experiments, adjacent sections of normal and treated chicken embryos were hybridized with a different probe such as that of jun. The dermis of control embryos showed a widespread c-jun expression (Fig. 1g and j). A similar pattern was found 8 h after PBS treatment (Fig. 1h and k). In contrast, in hydrocortisone-treated embryos, c-jun expression was significantly increased (Fig. 1i and l). This indicates that dermal cells responded to glucocorticoids by an increase in c-jun mRNA content. The same results were obtained in embryos treated for 24 h (not shown). We also observed a characteristic labeling in the spinal motoneurons of the neural tube (Fig. 1j) that remained unchanged after hydrocortisone treatment (Fig. 1).

Hydrocortisone decreases p54 and increases p68 mRNA expression within the dermis

The chicken c-ets-1 encodes, by alternate promoter usage, two nuclear proteins, p68 and p54. Therefore, we investigated the expression of the two c-ets-1 transcripts.

The p54 transcripts were homogeneously distributed whereas the p68 transcripts were found only in a subpopulation of the dermal cells, also confirming previous results (Quèva et al. 1993). The amount of p54 transcripts, well expressed in the dermis of control chicken with (Fig. 2b, e and h) and without PBS (Fig. 2a, d and g) decreased 8 h after hydrocortisone treatment (Fig. 2c, f and i). Decreased amounts of p54 transcripts were observed all over the dermis (compare Fig. 2h and i). P54 expression also decreased in neural tube and in the few blood vessels still present in the hypodermis (compare Fig. 2b and c). Conversely, the p68 transcripts appeared apparently unchanged in treated dermis (compare Fig. 2m-n and o). The p68 signal was more diffuse in the treated dermis than in control tissue where p68 expression followed a dotted pattern, restricted to some groups of cells. Hydrocortisone treatment seems to recruit more cells, but does not increase the signal in each expressing cell.

Hydrocortisone modulates p54c-ets-1 and p68c-ets-1 protein expression in dermis

Next, it was interesting to determine whether the same modulation of c-ets-1 transcripts observed by in situ hybridization was observed at the protein level in vivo as well as in vitro. Protein extracts were prepared from skin pieces peeled off from the back of chicken control and hydrocortisone-treated-embryos. Specific proteins were then immunoprecipitated with antibodies anti-EtsA, anti-β and anti-Ct (see Materials and Methods).

The antisera anti-ets-A and anti-β immunoprecipitated p68c-ets-1 protein preferentially in the treated dermis 8 h after treatment. In contrast, less p54 was detected in treated dermis than in normal dermis (Fig. 3 lanes 1 and 2; 7 and 9). Thus, in line with the in situ hybridization experiments, hydrocortisone treatment caused a reduction of p54 at protein level. Similarly, the increased amount of p68 transcripts was reflected at the protein level (Fig. 3 lanes 1 and 2; 5 and 9; 6 and 10). In control experiments, immunoprecipitations performed with antibodies against c-Jun showed an increased amount of p39 in treated tissues compared to the control (Fig. 3 lanes 3 and 4; 8 and 12). Surprisingly, when chicken skin cells were grown in culture, no modulation of c-Jun (Fig. 4 lanes 1, 5, 9 and 12) or c-Ets protein expression was detected (Fig. 4 lanes 2-4, 6-8, 10-11 and 13-14). This indicates that glucocorticoid effects on c-jun and c-ets-1 expression are specific to a peculiar population of competent dermal cells only found in a definite spatio-temporal context.
The expression of the 72 kDa type IV collagenase in dermis is decreased by hydrocortisone

Transverse sections of control chicken embryos at E6.5 and E7 and embryos treated with hydrocortisone for 8 h and 24 h were hybridized with an antisense 35S RNA probe specific to the 72 kDa type IV collagenase gene. As shown in Figure 4a and c, the 72 kDa type IV collagenase was widely expressed in the dermis of E6.5 and E7 chick, while the epidermis was negative. In treated embryos, 72 kDa type IV collagenase expression decreased in the dermis (Fig. 5b and d) although weak expression persisted in the feather buds which managed to grow under hydrocortisone treatment. Taken together, these results indicate that glucocorticoids decrease 72 kDa type IV collagenase expression in correlation with the p54c-ets-1 expression.

Discussion

Hydrocortisone can partially or totally inhibit the normal development of feathers in the chicken embryos (Sengel and Züst, 1968; Züst, 1971; Stuart et al., 1972). In the present study, we show that hydrocortisone treatments dramatically decrease p54c-ets-1 levels while they increase both p68c-ets-1 and c-jun expression. Furthermore, we found that the expression of p54c-ets-1 correlates with that of the matrix-metalloproteinase 72 kDa type IV collagenase.

Taken together, our observations raise the question of how glucocorticoids modulate collagen synthesis, degradation or both, and whether c-ets-1 takes part in this regulation.

A first hypothesis is that collagen accumulation results from increased synthesis. It has been reported that hydrocortisone increased mRNA levels of type I and V collagen genes in fibroblasts from human fetal dermis (Russell et al., 1989). It is interesting that the promoter of the α1 chain of the type I collagen contains putative binding sites for c-ets-1 (EBS) (Bornstein et al., 1987). However, whether c-ets-1 plays a role in the collagen gene transcriptional regulation remains to be explored. It is worth noting that an alternative usage of c-ets-1 promoters gives rise to two transcripts encoding two distinct proteins (Crepieux et al., 1993). Therefore, it has to be determined which transcript was down-regulated. In situ hybridization experiments with specific probes allowed us to show that hydrocortisone modulates in vivo the p54 and p68 messengers in chicken dermis.

We observed that the more abundant transcript, the p54 encoding messenger, strongly decreased in treated chicken dermis, and that the less abundant p68 transcript, in contrast, appeared to increase in treated dermis. Similar results were also observed at the protein level: the amount of p68 was increased and p54 became undetectable after treatment. This is interesting because the two c-ets-1 proteins have different transactivation domains and transactivating properties (Waslyky et al., 1991).

P68c-ets-1 was shown to be a more potent activator of the rat stromelysin promoter than p54c-ets-1 (Waslyky et al., 1991). We have shown in this report that the expression of p54c-ets-1 decreases at mRNA and protein level in treated chicken dermis. Similarly, 72 kDa type IV collagenase expression decreased in hydrocortisone treated dermis. Therefore, it is tempting to speculate that p54c-ets-1 could specifically regulate the 72 kDa type IV collagenase gene and the p68c-ets-1 the collagen gene.

A second hypothesis would link collagen accumulation to an impaired degradation by endogenous collagenase. It has been shown that the human collagenase1 and stromelysin1 promoters are activated by two Ets-family members, Ets1 and Ets2, via functional EBS (Ets-binding sites) (Gutman and Wasylyk, 1990; Buttìcè and Kurkinen, 1993). Recently, it was demonstrated that different members of the Ets-family can differentially regulate collagenase1 and stromelysin1 gene activity, in an additive or antagonistic fashion (Buttìcè et al., 1996). This suggests that probably there is a specific regulation of the matrix-metalloproteinases by the different Ets-family members. It is intriguing that we find a correlation of expression between the p54c-ets-1 and the 72 kDa type IV collagenase, suggesting that the p54, but not the p68c-ets-1, might be involved in the regulation of 72 kDa type IV collagenase. It will be interesting to find out whether the chicken promoter of the 72 kDa type IV collagenase contains functional EBS and is inducible by the p54c-ets-1 product. However, we should note that although several matrix-metalloproteinases contain in their promoter region EBS and AP1 site, the human 72 kDa type IV collagenase promoter apparently lacks these binding motifs (Huhtala et al., 1990; Gaire et al., 1994). In addition, we cannot exclude that the decrease of 72 kDa type IV collagenase expression in hydrocortisone treated embryos is due to a different mechanism. For instance, glucocorticoids are known to inhibit matrix-metalloproteinases expression (Olfringa et al., 1988; Quinones et al., 1989). The repression takes place at the AP1 site of the collagenase1 promoter via a physical interaction of...
the glucocorticoid receptors with the Fos and Jun proteins (Jonat et al., 1990; Lucibello et al., 1990; Schule et al. 1990). Interestingly, we found that c-jun was strongly expressed in control dermis and its expression increased at mRNA and protein level in the hydrocortisone treated dermis. Whatever the mechanism may be by which hydrocortisone regulates 72 kDa type IV collagenase, it is intriguing that hydrocortisone treatment of chicken embryos results in (1) inhibition of feather formation; (2) interstitial collagen accumulation; and (3), as observed here, a decreased 72 kDa type IV collagenase and p54c-ets-1 expression. Therefore, it is possible that the collagen accumulation by hydrocortisone treatment is a result of a diminished collagenolytic activity due to decreased expression of 72 kDa type IV collagenase. This possibility is supported by recent results showing that 72 kDa type IV collagenase is able to cleave, in addition to type IV collagen and fibronectin, interstitial collagen with the same kinetics than that of the interstitial collagenase or collagenase 1 (Aimes and Quigley, 1995). In conclusion we have found a link in the expression of a transcription factor, Ets1, and the matrix-metalloproteinases, 72 kDa type IV collagenase in the derma. Moreover we have pointed out that in vivo subtle mechanisms might take place to induce either the network of collagen fibers used by migrating cells in normal morphogenesis or under glucocorticoids treatments.

Materials and Methods

Biological model and tissue preparation

Eggs of the white Sussex chicken strain were incubated at 39°C in a humidified air chamber. E0 indicates the first day of incubation. At E5.5, chicken embryos were injected with 0.1 mg hydrocortisone (hydrocortisone 21 sodium succinate; Sigma) in 0.1 ml of PBS, whereas control embryos received 0.1 ml of PBS. The drug or control solutions were deposited on the chorioallantoic membrane at the level of the vascular area edge. Control and treated embryos were taken at E6.5, at E6.5 + 8 h and at E7.5. They were next fixed at 4°C for 16 h in 4% paraformaldehyde in PBS, dehydrated, embedded in paraffin and serially cut (7 μm). Transverse sections of the dorso-lumbar region were transferred to aminopropyltriethoxysilane (TESPA; Aiden)-coated slides and incubated at 42°C for 2 days. Slides were stored at 4°C until use.

Probes

For c-ets-1 transcripts, we used a common probe derived from a 750-bp BglII-HindIII fragment of the E26 provirus (Leprince et al., 1983). Antisense and sense RNA probes specific for the transcript encoding p54 were synthesized from a 140-bp EcoRI-ClaI fragment subcloned into pSP64 and pSP65 (Promega Biotech). This fragment contains the 5′ untranslated region and exon I 54 of the p54c-ets-1 (Dulguerov-Coquillat et al., 1988). Similarly, a 190-bp EcoRI-PstI fragment from pSp65c-ets-1 cDNA encompassing exons a and b (Leprince et al., 1988) was used as a template to synthesize the probe specific for the transcript encoding p68c-ets-1. The jun probe was synthesized from a 935-bp EcoRI fragment coming from the v-jun sequence (kindly provided by B. Waswylk) and subcloned into pSP64. Specific RNA probes for the transcripts encoding p54 and p68 were transcribed from the digested plasmids using 200 mCi of α-35S CTP and 200 mCi of α-35S UTP. Riboprobes for jun and ets were labeled with 200 mCi of α-35S CTP only. The chicken 72 kDa type IV collagenase gene, kindly provided by M. Kurkinen (Yang et al., 1996), was transcribed to produce sense and antisense probes and 200 mCi of α-35S CTP were used for labeling.

In situ hybridization

The in situ hybridization protocol was a modification of the method of Cox et al. (1984) and was performed according to specific recommenda-

References


Acknowledgments

We thank M. Yang and M. Kurkinen for the chicken 72 kDa type IV collagenase cDNA, C. Queva for advice and S. Saule for critical reading of the manuscript. This work was supported by grants from the Association pour la Recherche sur le Cancer.


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