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Cellular Retinol-Binding Protein-1 Is Transiently Expressed in Granulation Tissue Fibroblasts and Differentially Expressed in Fibroblasts Cultured from Different Organs

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We have reported that cellular retinol-binding protein-1 (CRBP-1) is transiently expressed by arterial smooth muscle cells during experimental intimal repair (P. Neuville, A. Geinoz, G. Benzonana, M. Redard, F. Gabbiani, P. Ropraz, G. Gabbiani: Am J Pathol 1997, 150:509–521). We have examined here the expression of CRBP-1 during wound healing after a full-thickness rat skin wound. CRBP-1 was transiently expressed by a significant proportion of fibroblastic cells including myofibroblasts. Expression started 4 days after wounding, reached a maximum at 12 days, and persisted up to 30 days when a scar was formed. After wound closure, most CRBP-1-containing fibroblastic cells underwent apoptosis. We have further investigated CRBP-1 expression in rat fibroblasts cultured from different organs. CRBP-1 was abundant in lung and heart fibroblasts and was detected in decreasing amounts in muscle, tendon, subcutaneous tissue, and granulation tissue fibroblasts. Dermis fibroblasts contained no detectable levels of CRBP-1. All-*trans* retinoic acid and transforming growth factor-β1 inhibited cell proliferation and increased CRBP-1 expression in fibroblastic populations except dermis fibroblasts. We demonstrate that during granulation tissue formation a subpopulation of fibroblastic cells express CRBP-1 *de novo*. We also demonstrate that CRBP-1 expression by fibroblasts is regulated *in vitro* by retinoic acid and transforming growth factor-β1. Our results suggest that CRBP-1 and possibly retinoic acid play a role in the evolution of granulation tissue. *(Am J Pathol 1997, 151:1741–1749)*

It is more and more accepted that fibroblasts from different tissues exhibit specific features that reflect differences in embryological origin, degree of differentiation, and/or functional activity (for review see Refs. 1–3). Among the criteria used to classify different fibroblastic cells, vitamin A storage,[4] cell surface markers,[3] and contractile or cytoskeletal protein expression[5] have proven very useful. It is also accepted that during wound healing or fibrocontractive diseases fibroblasts modulate into myofibroblasts[6–8] expressing features of smooth muscle (SM) cell differentiation. Myofibroblasts are most numerous when wound contraction takes place and disappear through apoptosis after wound closure.[9] We have recently shown that cellular retinol-binding protein-1 (CRBP-1) is transiently expressed by arterial SM cells during the intimal repair reaction after endothelial injury in rat aorta.[10] In the present study, we have investigated whether CRBP-1 is expressed in fibroblastic cells during granulation tissue formation after a full-thickness skin wound in the rat and in fibroblasts cultured from different organs and from granulation tissue. As retinoids exert significant effects on a variety of cellular processes including growth and differentiation[11] and as transforming growth factor (TGF)-β has been shown to play an important role during granulation tissue evolution (for review see Ref. 12), we have also evaluated whether CRBP-1 expression in different fibroblastic populations is modulated *in vitro* by all-*trans* retinoic acid (tRA) and TGF-β1.

Materials and Methods

In Vivo *Experimental Procedures*

A total of 75 adult female Wistar rats (body weight, 200 to 400 g) were used to produce wounds. The experiments were approved by the Ethical Committee of Geneva Medical Faculty. Five rats per time point were used. After anesthetizing with an intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, IL), a 2- × 2-cm skin square was removed from the mid-dorsal surface. Granulation tissue samples subsequently were collected for immunohistochemistry at 4, 8, 12, 16, 21, 25, and 30 days and for Northern blot hybridization and Western blot analysis at 7, 10, 12, and 15 days.

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Immunohistochemistry and in Situ *End Labeling*

Staining was performed according to previous studies.[10] Double staining was performed for CRBP-1 and α-SM actin or apoptosis. Briefly, granulation tissues were fixed in 4% buffered formaldehyde and embedded in paraffin. After deparaffinization and rehydration, 4-μm sections were treated with 0.5% H_2O_2 in methanol for 10 minutes at room temperature to inhibit endogenous peroxidase. After two microwave treatments of 5 minutes each in 10 mmol/L citrate buffer, pH 6.0, sections were preincubated for 15 minutes in 10 mmol/L Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and 10% normal pig serum and then incubated for 60 minutes with polyclonal rabbit CRBP-1 antibody.[10] This was followed by a 30-minute incubation with a biotinylated pig anti-rabbit IgG antibody. After incubation for 30 minutes with horseradish-peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark), sections were treated with 0.5 mg/ml diaminobenzidine and 0.1% H_2O_2 for 10 minutes. They were then washed in phosphate-buffered saline (PBS) overnight at 4°C. After treatment for 15 minutes at room temperature with HCl, pH 2.2, and rinsing in distilled water, sections were incubated for 60 minutes with monoclonal mouse α-SM actin antibody.[13] After a 30-minute incubation with alkaline-phosphatase-conjugated rabbit anti-mouse IgG, sections were treated with fast red substrate for 5 minutes and rinsed in distilled water. They were then counterstained for 1 minute with hematoxylin and mounted with Aquatex (Merck, Darmstadt, Germany).

In situ end-labeling of DNA fragments for apoptosis was performed using the terminal deoxynucleotide transferase nick end-labeling (TUNEL) technique[10,14] after CRBP-1 staining. After treatment for 15 minutes at room temperature with a buffer containing 30 mmol/L Tris, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride in 0.5% BSA, pH 7.2, sections were incubated for 60 minutes with 20 U/ml terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and 0.01 mmol/L biotin-16-dUTP (Boehringer Mannheim) in the same buffer. After washing in PBS, sections were incubated for 30 minutes with horseradish-peroxidase-conjugated streptavidin (Dako), treated with 0.5 mg/ml diaminobenzidine, 0.1% H_2O_2, and 0.1% cobalt chloride, and mounted with Aquatex (Merck). Photographs were taken using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) with Ektachrome EPY-64T film (Eastman Kodak, Rochester, NY).

Fibroblast Culture and Treatment

Fibroblasts were isolated from explants of heart, lung, muscle, tendon, dermis, and subcutaneous tissue of normal adult female Wistar rats (body weight, 200 to 300 g). At least three animals were used for each cell preparation. Briefly, animals were sacrificed by cervical dislocation after enflurane (Ethrane, Abott Laboratories) anesthesia. Heart explants were from the left ventricle, lung explants were from the periphery of lower lobes, muscle explants were from soleus, tendon explants were from tail tendon, and dermis and subcutaneous tissue were taken from mid-dorsal skin. After washing in Eagle's minimal essential medium (HyQ, HyClone Europe, Erembodegem-Aalst, Belgium) containing 20 mmol/L Hepes buffer (Gibco, Basel, Switzerland), 1-mm^3 pieces were cut and placed in a 10-cm^2 Petri dish. They were allowed to attach to the plastic dish for approximately 5 minutes and then immersed in 10 ml of minimal essential medium supplemented with 10% fetal calf serum (Seromed, Biochrom KG, Berlin, Germany), 100 IU/ml penicillin, and 100 μg/ml streptomycin (HyQ, HyClone Europe). Explants were subsequently incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. Fibroblasts migrated from explants after 2 to 7 days, and 2 to 3 weeks later, explants were removed from Petri dishes. When cells were confluent after 3 to 4 weeks, they were trypsinized with 2 ml of 0.25% trypsin (Gibco) and passaged into 10-cm^2 Petri dishes at a 1:2 split ratio. Some aliquots of these cells were used as primary cultured cells. Confluent cells at the fifth passage (P5) as well as primary cultured (P0) cells were harvested for Western and Northern blot analyses.

Fibroblastic cells, including myofibroblasts, were also isolated, by explant technique, from granulation tissue and granuloma pouch. Cells derived from granulation tissue were isolated at 7 days (GT-7) and 12 days (GT-12) after wounding. Granuloma pouch was made by injection of 1% croton oil in corn oil,[6] and cells were isolated 20 days after oil injection. All investigated cells were routinely monitored for the absence of mycoplasma contamination.

Confluent cells at P5 were seeded at a density of $5.5 \times 10^3/cm^2$ and treated with tRA (Sigma Chemical Co., St. Louis, MO) or recombinant human TGF-β1 (Sigma) for 6 days. tRA was dissolved in pure ethanol at 1 mmol/L and diluted in complete medium at a final concentration of 1 μmol/L. The medium was changed every 2 days. An equal amount of ethanol was similarly used as control. TGF-β1 was dissolved in 4 mmol/L HCl containing 0.1% BSA and diluted in complete medium at a final concentration of 10 ng/ml. Four days after treatment, cells were photographed using a Zeiss phase contrast microscope. Cell counting was performed with a hemocytometer (Brand, Wertheim, Germany). Cells were then harvested for Western and Northern blot analyses.

Western Blot

Sample preparation and CRBP-1 or α-SM actin detection were performed according to previous studies.[10,13] For desmin immunoblotting, 80 μg of total protein was fractionated on a 5 to 20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) as previously described.[15] After blocking with 5% nonfat milk in 10 mmol/L TBS for 1 hour, the filter was incubated for 2 hours at room temperature with monoclonal mouse desmin antibody (Dako). It was washed in TBS three times for 5 minutes each and then

incubated for 1 hour with goat anti-mouse IgG conjugated with horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands) in TBS/0.01% Triton. The specific binding was enhanced by a chemiluminescent method using an ECL kit (Amersham, Zürich, Switzerland) and detected on Kodak X-OMAT film. Quantification was performed by means of densitometric scanning (Genofit, Geneva, Switzerland).

Northern Blot Hybridization

Total RNA was extracted from cultured cells and granulation tissue using TRI REAGENT (Sigma) according to the manufacturer's instructions. Equal amounts of total RNA were separated by electrophoresis on 1% agarose gel and then transferred onto a Hybond-N blotting membrane (Amersham) overnight in 20X standard saline citrate (SSC). Steady-state levels of specific RNA species were visualized by Northern blot hybridization.[16] CRBP-1 cDNA probe[10] was labeled with [^{32}P]dCTP (10 mCi/ml; Amersham) using a Megaprime DNA labeling kit (Amersham). Prehybridization and hybridization were performed for 4 and 16 hours, respectively, at 63°C in 5X SSC, 2.5% Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.1% SDS, and 100 μg/ml salmon sperm DNA. Radiolabeled DNA probe was used at a concentration of 10^6 cpm per lane. Membranes were then washed twice with 2X SSC/0.1% SDS for 15 minutes each at 63°C, and after washing, membranes were exposed to x-ray film (X-OMAT, Kodak) between intensifying screens for 2 days at −80°C.

Statistics

Data are represented as mean values and SD. The number of experiments (n) is indicated in the Results section. A Student's *t*-test for unpaired samples was used for statistical analysis. Significant differences were accepted for $P < 0.05$.

Results

CRBP-1 Expression in Granulation Tissue Fibroblasts

To examine the expression of CRBP-1 in granulation tissue, serial sections were stained for general morphology and immunohistochemical detection of CRBP-1, α-SM actin, or both. Histological examination of 4-day-wound tissue sections showed that the granulation tissue contained few fibroblasts, many inflammatory cells, and some neovessels. Immunohistochemistry revealed that CRBP-1 expression already was present 4 days after wounding and was restricted to fibroblastic cells (Figure 1a). At this time, α-SM actin staining was localized exclusively to pericytic or smooth muscle cells of small vessels (Figure 1b). Eight days after wounding, numerous fibroblasts were clearly positive for CRBP-1 but only infrequently for α-SM actin (Figure 1, c and d). At 12 days, there was widespread positive fibroblast staining for CRBP-1 and α-SM actin, and their expression reached a maximum (Figure 1, e and f). Double staining for CRBP-1 and α-SM actin at 12 days after wounding revealed co-localization of both proteins in many myofibroblasts (Figure 2a). The staining intensity declined thereafter, although α-SM actin decreased more rapidly than CRBP-1. At 30 days, when the wound was closed and re-epithelialization had occurred, the number of CRBP-1-positive fibroblasts was diminished compared with previous stages, and as expected, fibroblasts were negative for α-SM actin (Figure 1, g and h). Only pericytic and smooth muscle cells of blood vessels remained positively stained for α-SM actin.

As described previously,[9] apoptotic cells initially appeared at 8 days and were maximal at 21 days. Double staining for CRBP-1 and apoptotic cells was co-localized in several fibroblastic cells (Figure 2b).

Immunoblot and Northern blot analyses consistently showed CRBP-1 expression in granulation tissue at 7, 10, 12, and 15 days after wounding (Figure 3). In both cases, the strongest signal was obtained at 12 days. By densitometric scanning of immunoblots (five experiments per time), there were 1.9-, 2.7-, and 1.8-fold increases at 10, 12, and 15 days, respectively, compared with 7 days. Immunoblots for α-SM actin at the same time points showed that the strongest signal was obtained at 12 days as well (Figure 3A). α-SM actin expression increased 4.1-, 4.5-, and 4.4-fold at 10, 12, and 15 days, respectively, compared with 7 days.

CRBP-1 Expression in Fibroblasts Cultured from Different Organs

Previous reports support a phenotypic heterogeneity of fibroblastic cells.[2,3,17] Hence, we have investigated the level of CRBP-1 expression in cultured fibroblasts derived from different tissues. CRBP-1 was present at different levels in all fibroblastic populations examined with the exception of those from dermis (we performed at least three experiments per group; see Table 1). In heart and lung fibroblasts there was an increase of CRBP-1 protein expression between P0 and P5, of 3.4- and 18.1-fold, respectively (Figure 4A). The CRBP-1 signal of muscle and tendon fibroblasts was lower than the signal for heart and lung fibroblasts. In muscle and tendon fibroblasts there was 10.4- and 13.9-fold increase of CRBP-1 at P5 compared with P0, respectively. Fibroblasts from subcutaneous tissue as well as GT-7 and GT-12 cells expressed lower amounts of CRBP-1 at P5 compared with P0 (Figure 4B). The decrease was 2.7-, 6.8-, and 11.4-fold, respectively. Dermis fibroblasts contained no detectable levels of CRBP-1 at any time. As reported previously,[18,19] cultured fibroblasts expressed different amounts of α-SM actin according to their origin. All P5 fibroblasts contained increased amounts of α-SM actin compared with the P0 cultures albeit in different proportions (Table 1). The increase was 2.9-fold in lung fibroblasts, 1.2-fold in heart fibroblasts, 11.6-fold in muscle fibroblasts, 1.5-fold in tendon fibroblasts, 2.0-fold in dermis fibroblasts, and 7.4-fold in subcutaneous tissue fibroblasts, respectively. The signal for

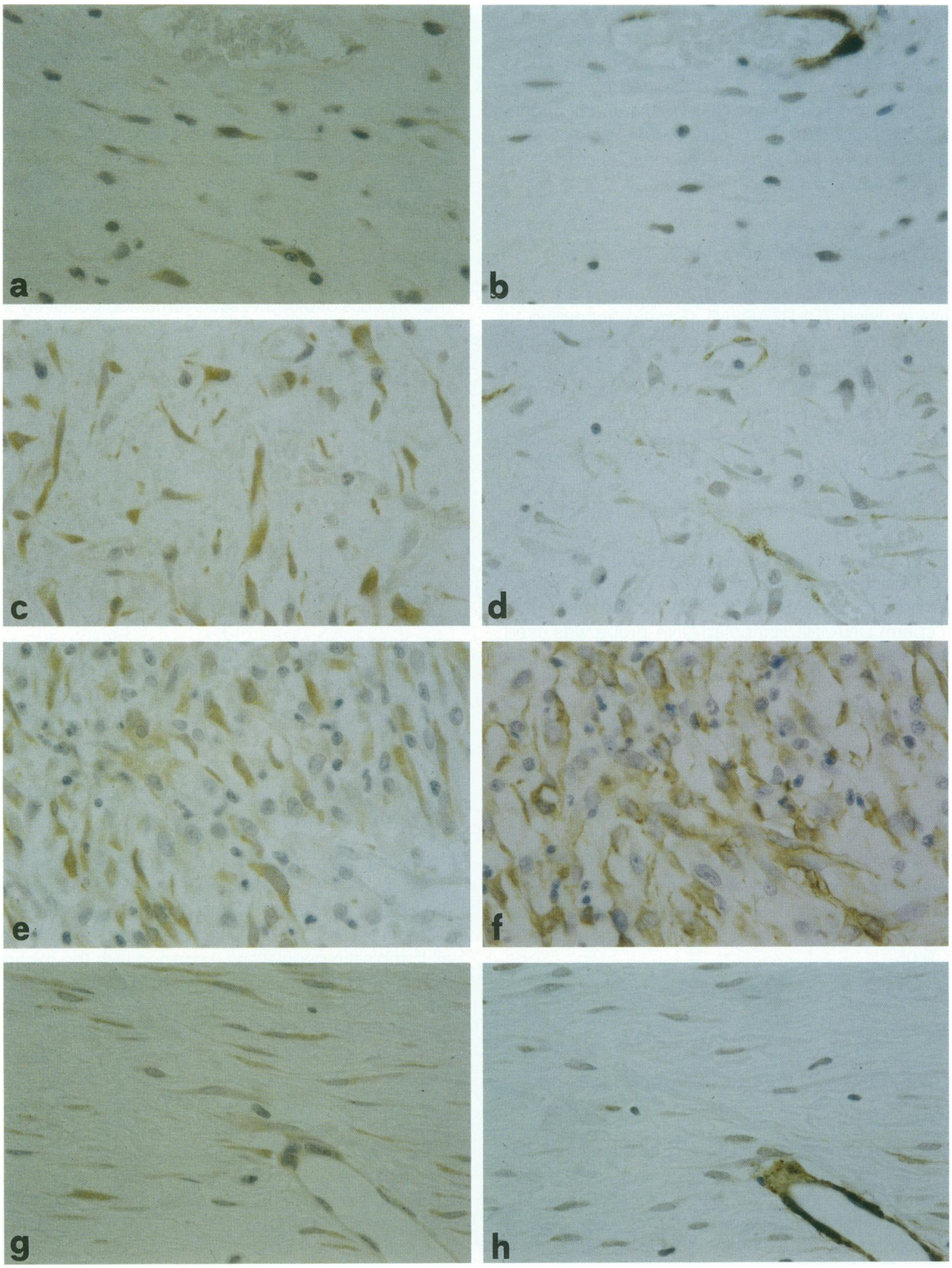

Figure 1. Immunolocalization of CRBP-1 (**a**, **c**, **e**, and **g**) and α-SM actin (**b**, **d**, **f**, and **h**) in granulation tissue fibroblastic cells after wounding. At 4 days, CRBP-1 expression is restricted to fibroblastic cells (**a**) and α-SM actin expression is exclusively present in vascular pericytic and SM cells (**b**). At 8 days, numerous fibroblastic cells are clearly positive for CRBP-1 (**c**), whereas only some are positive for α-SM actin (**d**). At 12 days, most fibroblastic cells stain for CRBP-1 (**e**) and α-SM actin (**f**). At 30 days, CRBP-1 expression persists in some fibroblastic cells (**g**) and α-SM actin expression is again present only in vascular pericytic and SM cells (**h**). Original magnification, ×630.

α-SM actin was very low in P0 cells isolated from granulation tissue at 7 and 12 days.

All tested fibroblasts at P0 and P5 were negative for desmin with the exception of lung fibroblasts (data not shown). The intensity of desmin signal in lung fibroblasts at P5 was 11.5-fold lower compared with cells at P0 ($n = 6$). Thus, fibroblasts from different origins were heterogeneous as far as CRBP-1, α-SM actin, and desmin expression is concerned.

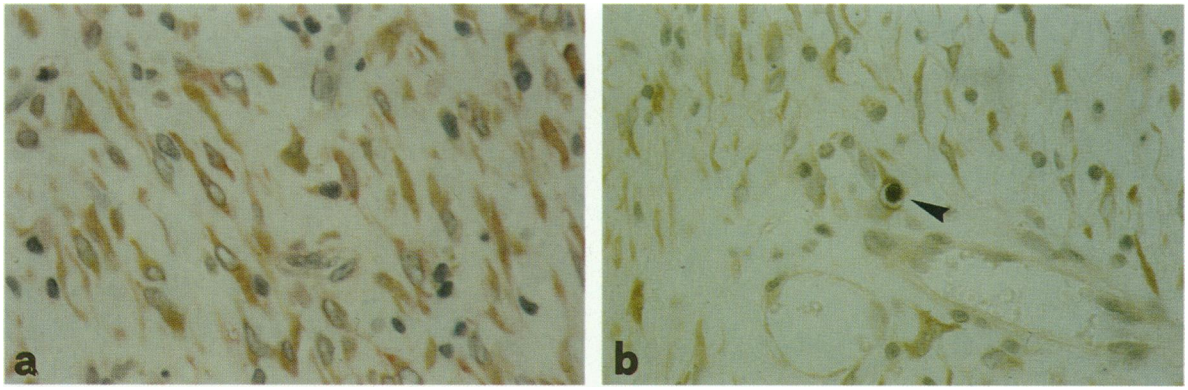

Figure 2. Double immunostaining for CRBP-1 and α-SM actin 12 days after wounding (**a**) and for CRBP-1 and apoptotic cells 16 days after wounding (**b**). In **a**, CRBP-1 and α-SM actin are co-localized in numerous fibroblastic cells and appear red-brown due to the mixture of peroxidase and alkaline phosphatase staining. In **b**, the nucleus of the apoptotic cell is black, whereas the cytoplasm of this cell as well as of those cells positive for CRBP-1 is brown (**arrowhead**). Original magnification, ×630.

CRBP-1 Expression after tRA and TGF-β1Treatment

It is well established that TGF-β1 influences the expression of α-SM actin in fibroblastic cells *in vivo* and *in vitro*.[20–23] We studied the effects of tRA and TGF-β1 in fibroblastic cells isolated from the aforementioned sources. In all cases, cell growth was markedly inhibited by a 6-day treatment with tRA or TGF-β1 (Figure 5). The cells became large and flattened, and an increase in number and thickness of stress fibers was induced as observed by phase contrast microscopy (data not shown). Cell shape and proliferation were not changed when fibroblasts were treated with ethanol as control (data not shown). tRA and TGF-β1, respectively, inhibited the proliferation of lung fibroblasts to 56.0 ± 10.4% (n = 5) and 53.7 ± 6.2% of control values (n = 5), of heart fibroblasts to 49.3 ± 11.0% (n = 3) and 42.0 ± 2.5% (n = 3), of dermis fibroblasts to 58.1 ± 2.9% (n = 6) and 56.0 ± 5.0% (n = 6), of subcutaneous tissue fibroblasts to 47.9 ± 1.4% (n = 4) and 44.8 ± 2.7% (n = 4), of muscle fibroblasts to 62.0 ± 3.2% (n = 3) and 54.6 ± 9.1% (n = 3), of tendon fibroblasts to 47.8 ± 3.7% (n = 3) and 37.8 ± 2.1% (n = 3), of cells derived from granuloma pouch to 67.0 ± 12.4% (n = 3) and 54.6 ± 6.4% (n = 3), and of cells derived from GT-7 and GT-12 to 73.7 ± 4.9% (n = 3), 69.7 ± 3.4% (n = 3), 76.7 ± 2.6% (n = 3), and 48.2 ± 1.8% (n = 3). TGF-β1 was more potent than tRA (Figure 5).

CRBP-1 was expressed in all tested cultured fibroblasts with the exception of those isolated from dermis. By means of Northern blot hybridization and Western blot analysis, we found that tRA and TGF-β1 stimulated CRBP-1 mRNA and protein expression in all CRBP-1-positive cells examined, albeit in different proportions. CRBP-1 expression after 6 days of tRA and TGF-β1 exposure was, respectively, increased 13.8-fold and 8.1-fold in lung fibroblasts (n = 4), 9.3-fold and 3.2-fold in heart fibroblasts (n = 2), 6.6-fold and 2.7-fold in muscle fibroblasts (n = 3), 7.8-fold and 4.4-fold in tendon fibroblasts (n = 3), 10.9-fold and 5.4-fold in subcutaneous

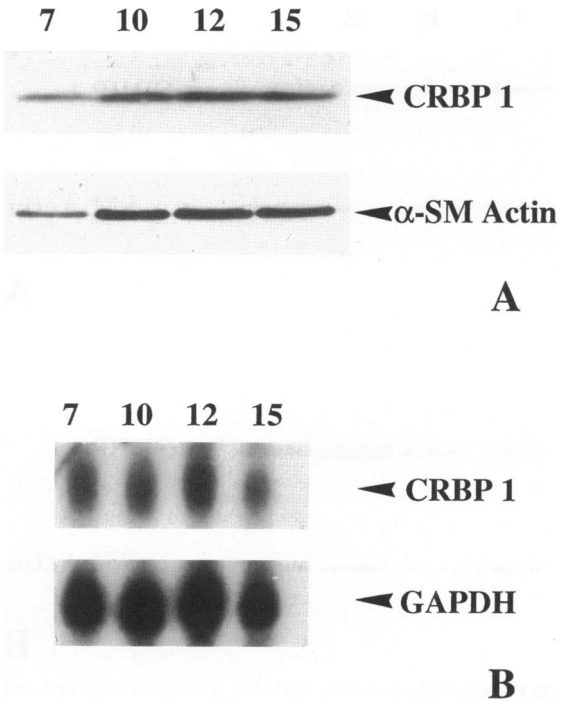

Figure 3. Western blot for CRBP-1 and α-SM actin at 7, 10, 12, and 15 days after wounding (**A**) and Northern blot for CRBP-1 expression in granulation tissue at 7, 10, 12, and 15 days after wounding (**B**). The strongest signal for CRBP-1 and α-SM actin is obtained at 12 days (**A**).

Table 1. CRBP-1 and α-SM Actin Levels in Cultured Fibroblasts Evaluated by Means of Western Blotting and Densitometric Scanning

Tissue	CRBP-1		α-SM actin	
	P0	P5	P0	P5
Lung	+/−	+++	+	+++
Heart	++	+++	++	+++
Muscle	+	++	+/−	+++
Tendon	+	++	++	+++
Dermis	−	−	++	+++
Subcutaneous tissue	++	+	+	++
Granulation tissue at 7 days	++	+/−	+/−	+
Granulation tissue at 12 days	++	+/−	+/−	++

−, no signal; +/−, traces; +, weak signal; ++, moderate signal; +++, strong signal. There were at least three experiments per group.

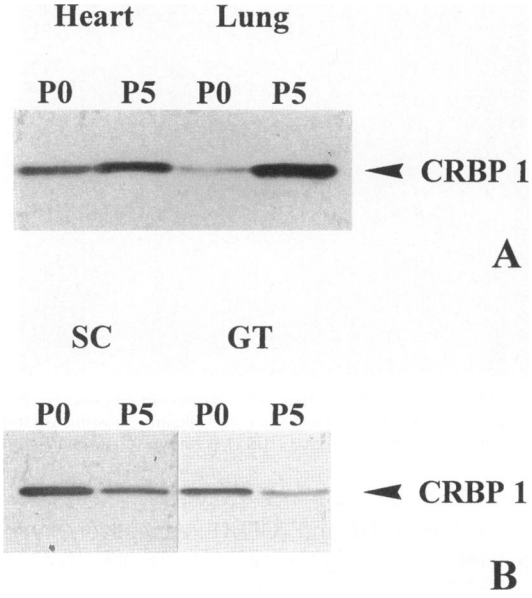

Figure 4. CRBP-1 expression evaluated by Western blot is increased at the fifth passage (P5) compared with the primary culture (P0) in fibroblasts derived from heart and lung (**A**) and decreased in fibroblasts derived from subcutaneous tissue (SC) and granulation tissue (GT) at 12 days (**B**).

tissue fibroblasts (n = 4), 7.3-fold and 6.8-fold in granuloma pouch fibroblasts (n = 2), 10.0-fold and 4.3-fold in GT-7 cells (n = 2), and 14.0-fold and 2.0-fold in GT-12 cells (n = 2) (Figure 6). Interestingly, stimulation by TGF-β1 was lower compared with tRA in all cases. TGF-β1 increased α-SM actin protein levels in all tested cells (1.7-fold to 2.9-fold; at least two independent experiments were performed for each cell type) with the exception of heart fibroblasts in which the level of α-SM actin was very high in control cells. Interestingly, α-SM actin expression in dermis fibroblasts, subcutaneous tissue fibroblasts, GT-12 cells, and granuloma pouch fibroblasts decreased to 79% (n = 4), 86% (n = 3), 83% (n = 3), and 63% (n = 2), respectively, on tRA treatment

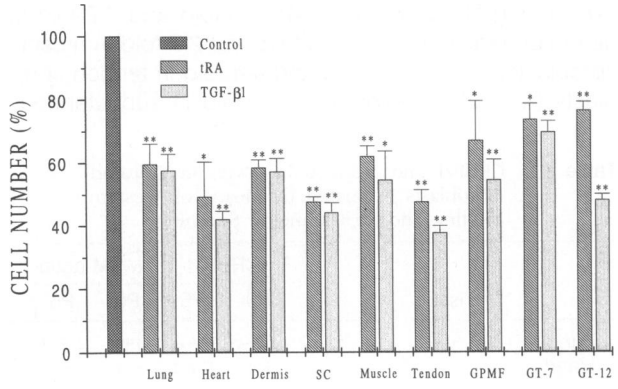

Figure 5. Histogram showing the proliferation evaluated by cell counting 7 days after plating of fibroblasts derived from lung, heart, dermis, subcutaneous tissue (SC), muscle, tendon, granuloma pouch (GPMF), and granulation tissue at 7 days (GT-7) and 12 days (GT-12) after wounding. The levels of cell proliferation inhibition are compared with 100% of control samples. Control, no treatment; tRA, 1 μmol/L tRA treatment; TGF-β1, 10 ng/ml TGF-β1 treatment. Ethanol treatment did not change cell proliferation, whereas both tRA and TGF-β1 always significantly decreased proliferation compared with controls ($^*P < 0.05$; $^{**}P < 0.01$).

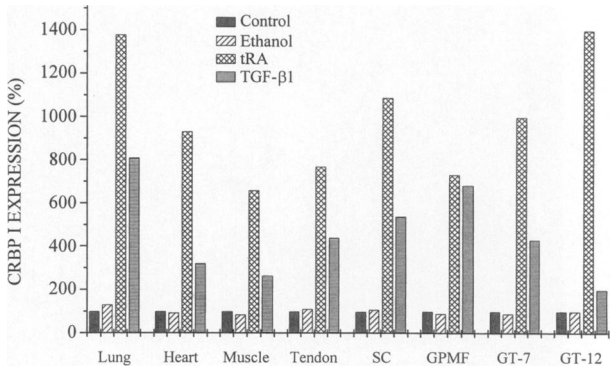

Figure 6. Histogram showing CRBP-1 expression in fibroblasts derived from lung, heart, muscle, tendon, subcutaneous tissue (SC), granuloma pouch (GPMF), and granulation tissue at 7 days (GT-7) and 12 days (GT-12) after wounding by laser densitometry of the autoradiograph of Western blots. The data represent mean values of two to four independent experiments. The expression in control cells is normalized to 100%. Control, no treatment; ethanol, ethanol vehicle control; tRA, 1 μmol/L tRA treatment; TGF-β1, 10 ng/ml TGF-β1 treatment. Both tRA and TGF-β1 increase CRBP-1 expression.

(Figure 7A). This effect did not occur in other fibroblasts (Figure 7B).

Discussion

The recent observation[10] that CRBP-1 is transiently expressed by arterial smooth muscle cells during the intimal repair reaction after endothelial injury of the rat aorta has suggested that this protein and therefore RA play a role in tissue repair. We have used here a more classical model, ie, the full-thickness skin wound, to examine the presence

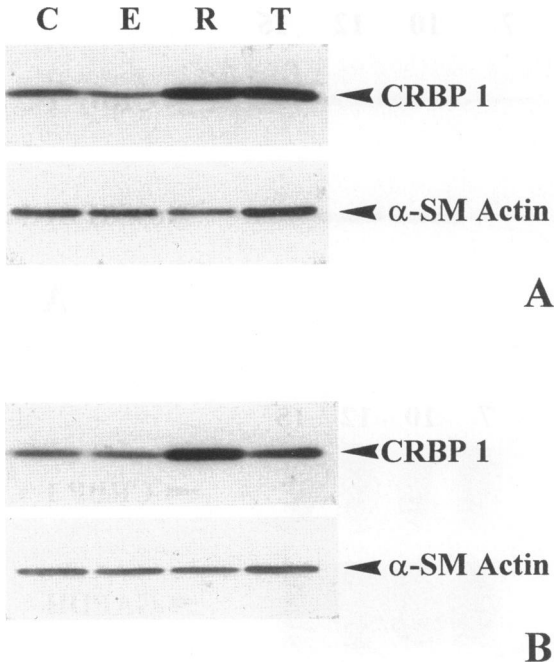

Figure 7. Western blot for CRBP-1 and α-SM actin in fibroblasts derived from granulation tissue (**A**) at 12 days after wounding (GT-12) and from lung (**B**). C, control; E, ethanol vehicle control; R, tRA; T, TGF-β1. tRA decreases α-SM actin expression in GT-12 but does not affect lung fibroblasts, whereas TGF-β1 increases α-SM actin expression in both cases. Both tRA and TGF-β1 increase CRBP-1 expression.

of CRBP-1 during granulation tissue evolution. As in arterial repair, CRBP-1 is transiently expressed by a subset of cells that appears very early in the healing process and disappears, at least in part through apoptosis, after wound closure. Most if not all these cells are myofibroblasts, as assessed by the presence of α-SM actin. The temporary expression of CRBP-1 by arterial SM cells during arterial repair and by myofibroblasts during skin wound healing supports the possibility that CRBP-1 and consequently retinoids play a role in these repair phenomena.

When grown *in vitro*, fibroblasts from different origins exhibit different growth properties despite their stereotyped morphology.[5,24] For example, cultured fibroblasts can modulate into myofibroblast-like cells.[25] It is well established that myofibroblasts (as well as fibroblasts) are heterogeneous as far as expression of cytoskeletal proteins such as vimentin, desmin, α-SM actin, and SM myosin heavy chain is concerned.[1] We demonstrate here that CRBP-1 is expressed in different amounts in fibroblasts cultured from several locations. During subculture, when it is expressed, CRBP-1 is either increased (eg, in lung, heart, muscle, and tendon fibroblasts) or decreased (eg, in subcutaneous and granulation tissue fibroblasts) compared with primary culture. Interestingly, cultured dermis fibroblasts contain no detectable levels of CRBP-1 at any time. In all cases, α-SM actin is increased. Presently, we have no explanation for these different responses, but they possibly correspond to different functional activities of fibroblastic cells from different locations. The comparable behavior of fibroblasts cultured from granulation tissue at 7 or 12 days after wounding with those cultured from subcutaneous tissue reflects the origin of most granulation tissue fibroblasts from subcutaneous tissue in our experimental model. These results reinforce the concept of fibroblast heterogeneity.[1–3]

tRA is a potential regulator of a large number of genes that may play a role in situations where myofibroblasts are involved. Among them, cellular fibronectin isoforms, which have been reported as a potential inducer of the myofibroblastic phenotype,[26] are down-regulated in fibroblasts treated by tRA.[27] Matrix metalloproteinases are also target genes of tRA and are important mediators of cell proliferation and movement. Recently it has been shown that tRA up-regulates stromelysin-3 in myofibroblasts of the stroma reaction to mammary carcinoma[28] whereas it down-regulates matrix metalloproteinases in other cell types.[29,30] This further implicates tRA in the overall wound healing process. CRBP-1 expression appears useful for the classification not only of the different myofibroblasts but also of fibroblasts from many sources, although not dermis, which may also reflect their responsiveness to retinoids.

Mesenchymal cells may be more responsive to retinol than to tRA.[31] Nonetheless, we choose to compare the response of fibroblasts from different sources to tRA to be sure that the effects we observed were not affected by the ability of retinol to affect intracellular levels of CRBP-1. Additionally, the conversion rate of retinol to tRA may vary from one fibroblast type to another. tRA is one of the mediators of vitamin A action, and its direct effect on the nucleus ensures that cells in all cases were subjected to the same concentration of retinoid.

It has been shown that tRA-mediated CRBP-1 up-regulation is a direct effect of tRA on gene transcription that is elicited without any new protein synthesis and is mediated through a direct binding of the Retinoic Acid Receptor α-Retinoid X Receptor α (RARα-RXRα) complex to the RA-responsive element of the CRBP-1 promoter.[32,33] This may well be the explanation for our *in vitro* results. At the same time, CRBP-1 is not up-regulated by tRA in dermis fibroblasts, indicating that such a proposed mechanism does not universally apply.

TGF-β1 transcriptionally stimulates the expression of RARα and RXRα genes in an osteoblastic cell line.[34] It is conceivable that the same mechanisms of RARα-RXRα transcriptional activity stimulation plays a role in fibroblasts. These effects of TGF-β1 also point to an important relationship with tRA.

It has been suggested before that there are interactions between tRA and TGF-β1.[35] We show here that tRA and TGF-β1 significantly inhibit proliferation of all investigated fibroblasts *in vitro*. This is consistent with previous findings from studies of a variety of cell types.[36–38] Expression of active TGF-β isoforms occurs directly or indirectly after tRA treatment in a wide variety of cell types, such as human tumor cell lines,[39–41] mouse keratinocytes,[42] and rat prostatic epithelial cells.[43] tRA up-regulates TGF-β1 and TGF-β2 in rat and mouse mesenchymal stem cell lines[44] and TGF-β2 in rat kidney fibroblasts and human lung carcinoma cells.[45] In these systems, the induction of TGF-β1 expression is post-transcriptional; however, sequences that mediate tRA activation recently have been identified in the TGF-β1 gene promoter although a typical RA-responsive element was not found.[41] The ability of tRA to inhibit rat NRP-152 cell growth was blocked more than 95% by a monoclonal antibody raised against TGF-β1.[43] Using TGF-β1 antisense oligonucleotides or TGF-β1 neutralizing antibodies, Turley et al[46] demonstrated that the expression of autocrine TGF-β1 in HL-60 cells induced by tRA was partially blocked. These observations suggest that tRA-induced fibroblast growth arrest depends in part on the increase of autocrine TGF-β. This may not be the case for all cell types however, as in chicken embryo fibroblasts, tRA treatment has no effect on TGF-β secretion.[47]

We have observed that tRA is able to both inhibit fibroblast proliferation and increase CRBP-1 at the same time whereas *in vivo* CRBP-1 is increased when cell proliferation is elevated. The same issue exists for TGF-β1: *in vitro* TGF-β1 reduces cell proliferation and up-regulates α-SM actin, whereas *in vivo* TGF-β1 is present when α-SM actin is accumulating but when cell proliferation is maximal. There could be several explanations for these differences. For example, many tissue factors and matrix elements that may modulate the effects of tRA and TGF-β1 simply are not present *in vitro*. Furthermore, these differences also may be due to differences in the concentration of tRA and TGF-β1 *in vivo* and *in vitro*.

As retinoids and TGF-β1 have been implicated in biological events related to myofibroblast evolution, we ex-

pected that both agents similarly would affect CRBP-1 and α-SM actin expression in cultured fibroblasts. Our results show that this is only partially the case. Both tRA and TGF-β1 induce growth inhibition and up-regulate CRBP-1 protein and mRNA. This is the first report showing that TGF-β1 increases CRBP-1 synthesis, although additional studies are needed to extend these observations to *in vivo* situations. tRA and TGF-β1, however, affected α-SM actin expression differently; TGF-β1 always up-regulated, whereas tRA never up-regulated and even decreased α-SM actin expression in several fibroblastic populations. These results suggest a partial converging action of tRA and TGF-β1 on signaling pathways that affect fibroblast phenotypic modulation rather than an effect of tRA solely mediated by synthesis and secretion of TGF-β1.

Fibroblast activation is a limiting step for granulation tissue formation[48] and is influenced by various agents.[1] Among these, TGF-β1 plays a fundamental role in wound healing[49,50] and is involved in the phenotypic modulation of fibroblasts during granulation tissue formation.[20,51] It is tempting to speculate that the early *in vivo* expression of CRBP-1 followed by α-SM actin is also due to TGF-β1. TGF-β1 is present early after wounding.[52] Our *in vitro* experiments show that TGF-β1 increases both CRBP-1 and α-SM actin in subcutaneous tissue fibroblasts, whereas tRA increases CRBP-1 but down-regulates α-SM actin expression in the same cells. It is noteworthy that at later stages most fibroblastic cells retain the capacity of expressing CRBP-1 in the period during which myofibroblast apoptosis is occurring. This sustained expression may be due to tRA inasmuch as at this time α-SM actin starts to disappear. Moreover, it is well known that the presence of CRBP-1 may increase tRA content by stimulating oxidation of vitamin A.[53,54] It has also been demonstrated that retinoids are responsible for apoptosis production in several cell types.[55,56] This is compatible with the possibility that retinoids induce genes involved in programmed cell death of myofibroblasts. Overall, our findings suggest that the early increase of CRBP-1, probably by TGF-β1 stimulation, can be considered as a marker of activation and differentiation of granulation tissue fibroblasts and that the accumulation of CRBP-1 may increase tRA, which consequently may then modulate the late evolution of granulation tissue. This is consistent with the aforementioned findings that tRA may down-regulate expression of proteins, such as cellular fibronectin isoforms,[27] that are inducers of the myofibroblastic phenotype.[26] It would be of interest in these regards to study the possible presence of CRBP-1 in myofibroblasts during human normal and pathological wound healing or during other situations such as fibroses and stroma reaction to epithelial tumor where myofibroblasts have been shown to be present and to play an important role in retraction phenomena and in extracellular matrix production.[57]

In conclusion, we show here that CRBP-1 is a marker of activation and differentiation of fibroblastic cells during granulation tissue formation. CRBP-1-containing fibroblasts appear to accumulate actively during the initial steps of wound repair and disappear later through apoptosis. Thus, CRBP-1 expression reflects fibroblast heterogeneity and is a useful additional marker for the classification of different myofibroblastic subsets. *In vitro* studies indicate that tRA stimulates CRBP-1 production by fibroblasts from many but not all sources and that TGF-β1 exerts a similar effect. Our results suggest that CRBP-1 and tRA play a role in the evolution of granulation tissue. This knowledge may be useful to design agents with the potential of influencing pathological wound healing as well as fibrocontractive diseases.

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