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Expression of the gene of the α-smooth muscle-actin isoform in rat liver and in rat fat-storing (ITO) cells*

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Summary. Fat storing cells (FSCs) in the liver represent the main site of vitamin A deposition in the body. These cells are considered to play an important role during scar formation and fibrogenesis in the liver. The putative descent of FSCs from the fibroblastic or from the myofibroblastic system have not been determined yet by morphological or immunohistochemical studies. To further define the origin of these liver cells, we analysed the pattern of expression of three structural proteins: vimentin, desmin and the α-smooth muscle (SM)-actin isoform in FSCs of the rat liver, in smooth muscle cells (SMCs) from the aorta and in rat skin fibroblasts. FSCs were studied by immunohistochemical methods immediately after isolation, at days 3 and 7 after plating. FSC-gene-expression was also analysed by Northern blot analysis of total RNA extracted from cells in culture at days 3 and 7 after isolation. Arterial SMCs and skin fibroblasts were studied in a similar way. For comparison, isolated rat hepatocytes and Küpffer cells (Kc) were studied. Of freshly isolated FSCs, 100% were vimentin-positive, 50% were desmin-positive, but all were α-SM-actin negative. Three days after isolation, FSCs were clearly positive for vimentin and desmin and weakly α-SM-actin-positive, as demonstrated by indirect immunofluorescence as well as by the immunoperoxidase technique. Desmin, α-SM-actin and vimentin staining was further increased at day 7 after isolation, and α-actin specific transcripts in FSC-RNA were clearly detectable at day 7 after isolation. Passaged arterial SMCs were vimentin- and α-SM-actin-positive, but desmin-negative and fibroblasts were only vimentin-positive. α-SM-actin isoform gene expression was strongly increased in the rat liver after acute or chronic damage induced by treatment with carbon tetrachloride. Positive staining was detected in cells which were also desmin-positive. As FSCs are vimentin-, desmin- and α-SM-actin-positive, we suggest that they are smooth muscle-related myo-fibroblasts with special functions.

Key words: Liver fibrosis – ITO-cells – α-actin

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Introduction

Four distinct main types of non-parenchymal cells are recognized in the liver: Kupffer cells (liver macrophages), liver endothelial cells, fat storing cells and pit cells (natural killer cells of the liver). In the last two decades there has been increasing interest in fat storing cells (FSCs). Ito and Nemoto (1952) first recognized this cell population, which is characterized by large fat droplets in the cytoplasm. Wake (1971) showed that staining of stellate FSCs by the gold-chloride method was due to the reduction of gold chloride by vitamin A, and Hendriks et al. (1984) recognized that storage of vitamin A is the main function of FSCs.

FSCs are located in the space of Disse in close contact with hepatocytes on one side and with endothelial cells on the other.

McGee and Patrick (1972) first described perisinusoidal cells containing cytoplasmic lipid droplets (another name for FCSs) as responsible for the de novo synthesis of connective tissue in the acutely damaged murine liver. The results were confirmed and extended by Kent et al. (1976) in the rat. De Leeuw et al. (1984) first showed that isolated FSCs in culture contain collagen and divide. They furthermore showed the presence of vimentin, actin and tubulin in these cells. Discussion of the origin of FSCs has concentrated on whether they stem from fibroblasts or from smooth muscle cells. Although FSCs in culture showed microfilament bundles, the dense bodies around the microfilament bundles typical of myofibroblasts could not be detected (De Leeuw et al. 1984). In favor of the theory that FSCs derive from smooth

muscle cells was the finding of desmin in FSCs of the normal rat liver (Yokoi et al. 1984) as well as in CCl_4-induced liver injury (Burt et al. 1986). Furthermore, desmin-positive FSCs have been found to actively participate in scar formation after focal liver injury (Ogawa et al. 1985, 1986) and desmin has also been demonstrated in isolated FSCs (Tasutsumi et al. 1987). On the other hand, desmin has been demonstrated not only in skeletal and cardiac muscle (Lazarides et al. 1978), but also in non-muscle cells (Gard et al. 1979). In addition not all muscle cells express the desmin gene, which implies that desmin is not sufficient to identify a smooth muscle cell.

Actins are highly conserved proteins which are ubiquitous in eukariotic cells (Carroll et al. 1986). Amino-acid sequencing data allowed the identification of "cytoplasmic" and "muscle" actins (Carroll et al. 1986), and three isoforms of the muscle-α-actin have been identified: α-skeletal muscle, α-cardiac muscle and α-smooth muscle (Leavitt et al. 1985).

The availability of a cDNA probe specific for α-actin and of a monoclonal antibody specific for the smooth muscle (SM)- isoform of α-actin allowed us to demonstrate α-SM-actin gene expression in isolated rat FSCs during culture. By means of these two methods we could also show that FSCs are vimentin-, desmin- and α-SM-actin-positive in culture and differ from arterial passaged SMCs because the latter are vimentin- and α-SM-actin-positive, but desmin-negative, as well as from skin fibroblasts which are positive for vimentin but negative for both desmin and α-SM-actin. Hepatocytes are negative for all three cytoskeletal proteins and Kupffer cells are only vimentin-positive. Immunohistological studies in fibrotic rat livers demonstrate that α-SM-actin gene expression can be detected in the same area where desmin-positive cells are present, mostly in fibrous septa. Taken together our findings suggest that FSCs are myofibroblasts derived from smooth muscle cells, with special commitments including vitamin A-storage and scar-formation.

Material and methods

Reagents. Materials were purchased from the following sources: normal and methionine-free Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) from Flow (Bonn, FRG), collagenase from Clostridium hystolyticum from Boehringer Mannheim (Mannheim, FRG), pronase E from Merck (Darmstadt, FRG), collagenase type I (125-250 U/mg) and elastase type III (20 U/mg) from Sigma (Munich, FRG), nycodenz from Nyegaard and Co. (Oslo, Norway), penicillin/streptomycin and L-glutamine from Seromed (Berlin, FRG), bovine serum albumin (BSA), ethidium bromide and 2-mercaptoethanol, from Sigma (Munich, FRG), sodium dodecylsulphate (SDS) BIO-Rad Laboratories (Munich, FRG), guandinium isothiocyanate from Fluka (Bern, Switzerland), cesium chloride and agarose from BRL (Heidelberg, FRG). ^{32}P-labeled dCTP (spec. act. 700/Ci/mmol) and a nick-translation kit were purchased from Amersham Buchler GmbH (Braunschweig, FRG). The monoclonal antibody against α-smooth muscle-actin isoform was a gift from Dr. Skalli (Dept. of Pathology University of Geneva, Switzerland) (Skalli et al. 1986) and the monoclonal antibody against desmin as well as the peroxidase-labeled anti-mouse IgG were from Dako (Copenhagen, Denmark). Monoclonals ED 2 and ED 3 (Dijkstra et al. 1985) against macrophage antigens were a gift of Dr. Dijkstra (Dept. of Histology, Free University of Amsterdam, The Netherlands).

Cell isolations and cultures

Isolation of FSCs and other liver cells. FSCs were isolated from the liver of female BN/BiRiJ rats by the perfusion method according to De Leeuw et al. 1984) as described previously (Ramadori et al. 1987, 1989). FSCs were >95% pure as assessed by light microscopy (large fat droplets), autofluorescence (vitamin A content) and sudan red staining. Contaminating cells were mostly endothelial cells. No Kupffer cell (Kc) contamination was observed by staining freshly isolated fixed cells with the monoclonal antibody ED 2. Cells were plated on plastic or glass cover slips (2.5×10^5/ml/well) in 24-well Falcon plates. Culture medium (Dulbecco's modification of Eagle's medium) containing 15% fetal calf serum was changed for the first time 3 days after plating and thereafter every other day. Cells in culture were examined by phase contrast microscopy (Andus et al. 1987) fat staining (Ramadori et al. 1989) and by fluorescence microscopy (vitamin A autofluorescence) (Ramadori et al. 1989). Contamination of the culture with Kc was excluded in the same way as described above and contamination with endothelial cells was excluded by staining the culture with an antiserum against factor VIII. In any case, it is known that endothelial cells from rat liver normally survive only on specially coated culture plates (De Leeuw et al. 1982). Hepatocytes and Kc were isolated as described previously (Ramadori et al. 1983, 1986) by the perfusion method according to Seglen (1972) and Knook et al. (1977), respectively.

Cells were also plated on plastic or glass cover slips. The culture medium was DMEM with 2% bovine serum albumin for hepatocytes and DMEM with 10% FCS for Kc.

Isolation and culture of arterial smooth muscle cells (SMCs) and fibroblasts. Isolation of SMCs from pieces of the aorta taken from the same animal used for FSC isolation was performed according to Oakes et al. (1982) and Kocher et al. (1985). After scraping off the endothelium under microscopic control, pieces of aorta were digested in a solution containing collagenase (0.2% wt/v) and elastase (0.05% w/v) with continuous shaking for 1 h at 37° C. After digestion, cells were washed in DMEM (5 min, 400 g), analysed by trypan blue staining for viability, counted and plated on 24-well Falcon plates in DMEM containing 20% FCS (1×10^5/well/ml), or on 6-well Falcon plates (1×10^6 cells per well). For immunhistochemical studies, cells were plated on 24-well Falcon plates containing either glass or plastic coverslips. Skin fibroblasts were obtained after digestion of pieces of epidermis and dermis in a collagenase-containing solution for 30 min at 37° C. After digestion cells were washed three times with DMEM and plated either on 24-well Falcon plates (0.5×10^5 cells/well), on tissue culture plates, or on plastic or glass coverslips (5×10^4/ml/well). Arterial SMCs and fibroblasts were used for immunocytochemistry mainly after the first passage.

The culture medium was changed daily. FSCs, hepatocytes, Kc, arterial SMCs and fibroblasts were kept at 37° C in a 5% CO_2/95% atmosphere and 100% humidity.

RNA extraction and Northern Blot analysis. For RNA studies, cells were plated on 6-well Falcon plates. At different times after plating, cells were lysed with guandinium isothyocyanate and RNA was extracted from the lysates according to Chirgwin et al. (1979) as described elsewhere (Ramadori et al. 1985). RNA was then separated by agarose electrophoresis (Ramadori et al. 1985), blotted on nitrocellulose paper, prehybridized and hybridized using ^{32}P-labeled cDNA probes.

cDNA probes specific for α-actin (Schwartz et al. 1980), for rat α2-macro-globulin (Gehring et al. 1987), for murine (Kioussis

et al. 1981) or human albumin (Kurnit et al. 1982) and for human fibronectin (Umezewa et al. 1985) were labeled with ^{32}P-dCTP by nick translation. The alpha-actin-cDNA-probe contains nucleotide sequences common to beta- and gamma-actin (Schwartz et al. 1980) and allows the detection of beta- and gamma-specific mRNA (2.1 Kb) and α-actin-specific mRNA (1.6 Kb). Hybridisation was performed in plastic bags by incubation in a water bath at 42° C over night. Nitrocellulose filters were then washed under different stringency conditions, dried and incubated with an x-ray film at −70° C.

Induction of acute liver damage and fibrosis. Acute liver damage in Wistar rats (body weight about 200 gr) was induced by oral treatment with a carbon tetracloride/olive oil solution (50% v/v) according to Enzan (1985) but reducing the applied volume to 150 µl/100 g b.wt. Control animals were treated with oil alone. At 48 h after treatment, the animals were sacrificed and tissue samples were taken for histology. For induction of liver fibrosis, Wistar rats were treated orally once a week with carbon tetrachloride according to Proctor and Chatamra (1982). Animals were sacrificed after the tenth administration and the livers were removed for immunchemistry and light microscopy of formalin-fixed sections.

Immunhistochemistry. Freshly isolated FSCs and Kc were centrifuged directly on a coverslip in a cyto-centrifuge (5 min, 300 rpm for FSCs and 1300 rpm for Kc) and fixed with methanol (5 min) and acetone (10 sec at −20° C.

Cell cultures were fixed using the same method. Cells were then washed three times with phosphate-buffered saline (PBS) containing 0.1% BSA (PBS-BSA) and covered with rabbit serum for 20 min in order to reduce non-specific background. Cells were then incubated with the specific antibodies (50 µl) against vimentin, desmin, α-SM-actin, or Kc-specific antigens (1 h at 37° C) in a humid chamber. Cells were again washed three times with PBS-BSA and incubated with peroxidase-labeled IgG against murine immunoglobulins. The peroxidase labeled IgGs had been previously absorbed with rat serum to avoid nonspecific binding.

Cells were then washed and incubated with a diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01%) solution, washed, counter-stained in Meyers hemalum and washed again before covering with a coverslip. Some experiments were performed using FITC-labeled antibodies against murine immunoglobulins.

Cryostat sections (6 µm thick) of rat liver tissue (snap frozen in liquid nitrogen) were dried and fixed with cold (−20° C) acetone (10 min). Immunohistochemical studies were performed by means of the peroxidase-technique as described above.

Results

Immunocytochemistry of the different cells

Freshly isolated liver cells. Freshly isolated FSCs were clearly positive for vimentin, about 50% were positive for desmin, but all were negative for SM- α-actin. Freshly isolated Kc stained with the antibody against vimentin but were negative for desmin and α-actin (not shown). Differentiation between vimentin-positive FSCs and Kc were achieved by morphology, fat staining and monoclonal antibodies (only blood monocytes or Kc were stained with ED 1 or ED 2 respectively).

Cell cultures. In cultures of passaged arterial SMCs and fibroblasts confluence was reached within 3–7 days. Arterial SMCs were positive for vimentin and α-SM-actin but not for desmin; fibroblasts were positive only for vimentin (Table 1). FSCs cultures were studied at days 3, 5 and 7 after plating. Staining for α-SM-actin became slightly positive at day 3 and (Fig. 1 D) increased during the first 7 days in culture (Fig. 1 H). Also desmin (Fig. 1 C, G) and vimentin (Fig. 1 B, F) staining increased during time in culture. Seven days after isolation most of the cells were well spread and showed the typical vimentin desmin and α-SM-actin intermediate filaments (Fig. 1 F, G, H). Cells still contained fat droplets as demonstrated by fat staining (data not shown). No cells stained with the monoclonal antibody ED 2 could be detected, indicating that no contaminating Kc were present in the culture.

Table 1. Detection of vimentin, desmin and α-SM-actin in different hepatic and non hepatic cells by immunohistochemistry

	vimentin	desmin	α-SM-actin
FSCs	+	+	+
SMCs	+	−	+
Fibroblasts	+	−	−
Kc	+	−	−
Hepatocytes	−	−	−

Kupffer cell as well as hepatocyte cultures remained negative for desmin and α-actin up to day 7 of culture. Freshly isolated and cultured Kc were vimentin-positive.

Northern Blot Analysis

Total RNA extracted from rat skeletal muscle, rat cardiac muscle, rat skin fibroblasts rat hepatocyte cultures (at day 1 of culture without dexamethasone), and FSCs was run in an agarose gel, blotted on nitrocellulose and hybridized with albumin, α_2-macroglobulin, fibronectin and α-actin-specific-^{32}P-labeled- cDNA probes. α-actin specific mRNA (1.6 Kb) was detected in FSC-RNA samples (Fig. 2). Its size was similar to that of the transcripts from skeletal as well as cardiac muscle. Fibroblast- and hepatocyte-RNA contained only β- and gamma actin transcripts (2.1 kb) of similar size of those contained in the FSC-RNA: the provenience of the total RNA studied was further characterized by the presence of albumin and α_2-macroglobulin in hepatocyte-RNA, of α_2-macroglobulin and fibronectin mRNA in FSC-RNA and of fibronectin-mRNA in fibroblasts-RNA. That is to say only FCSs-RNA contains transcripts specific for alpha-, beta- and gamma-actin. α-actin gene-expression in isolated FSCs increases during the time of culture. In fact, when total RNA from FSCs at day 3 or day 7 after isolation was studied by northern blotting, α-actin transcripts were clearly detectable only at day 7, whereas beta- and gamma-actin transcripts were already detectable at day 3 of culture (Fig. 3).

Histopathology and Immunohistochemistry of liver tissues

Histopathology. Massive liver necrosis was observed after a single large dose of CCl_4. The necrotic areas

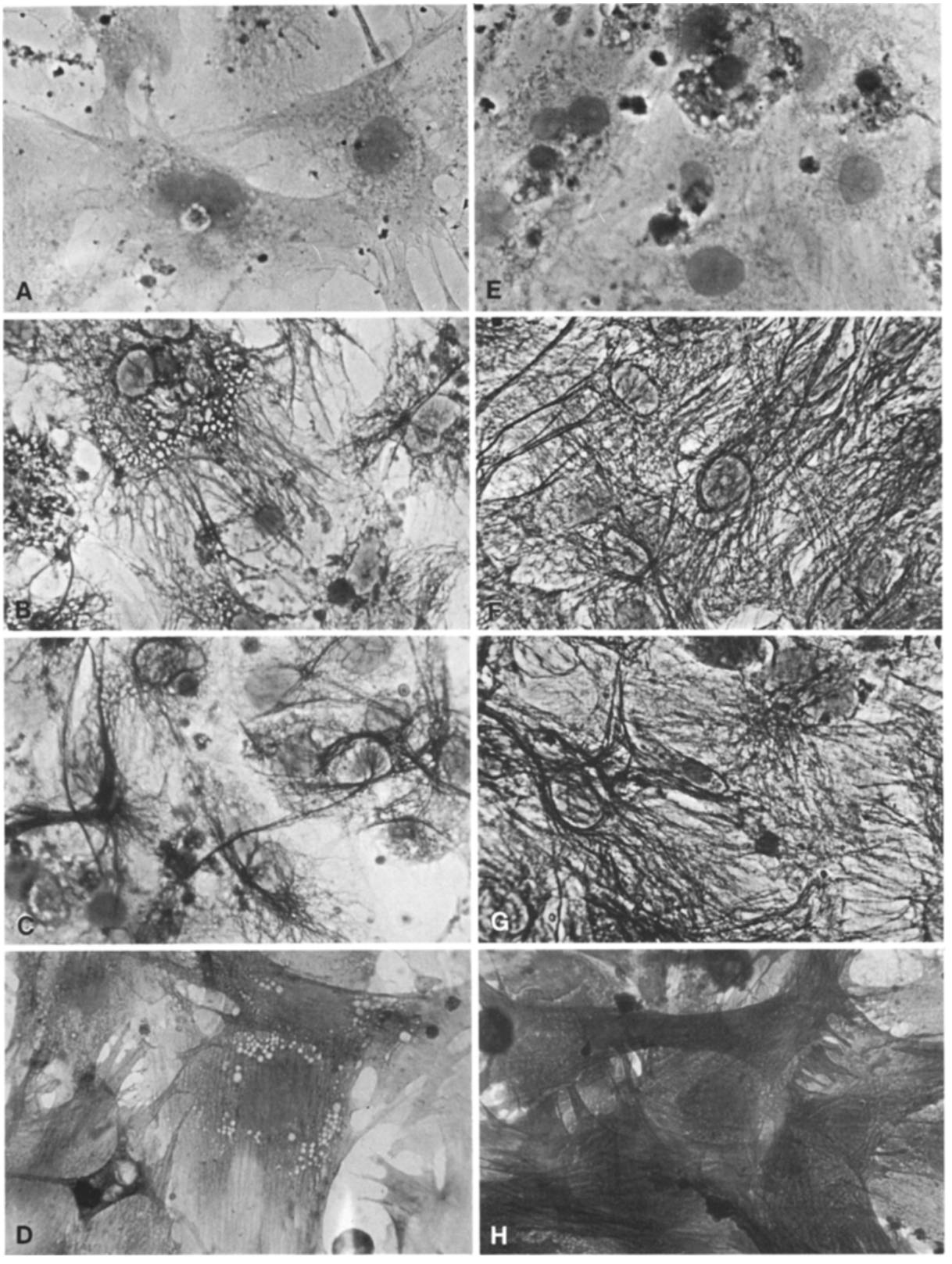

Fig. 1 A–H. Demonstration of intermediate filaments in cultured rats FSCs 3 **A–D** or 7 **E–H** days after plating. Cells were fixed (see material and methods) and stained for ED 3 (negative control), **A, E**, vimentin **B, F**, desmin **C, G** or α-SM-actin- **D, H** gene-expression. Phase contrast micrographs (×400, **A, B, C, E, F, G**) and light microscopy pictures (×400, **D, H**)

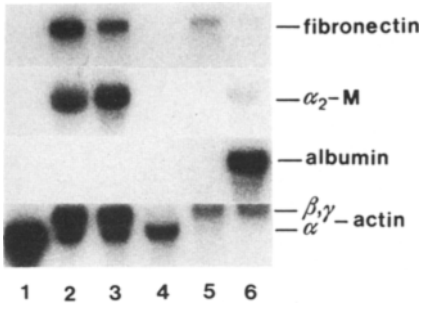

Fig. 2. FSCs of rat liver express the gene of α-SM-actin. Northern blot analysis of total RNA extracted from 1) rat skeletal muscle 2) rat FSCs 7 days and 3) 14 days after isolation, 4) rat heart muscle, 5) rat skin fibroblasts, 6) rat hepatocytes. RNA was hybridized with ^{32}P-labelled cDNA-probes specific for chicken α-actin, for fibronectin, for α_2-macroglobulin and for albumin

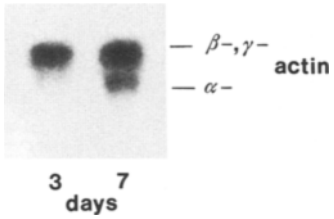

Fig. 3. Northern Blot Analysis of total RNA extracted from FSCs lysed at day 3 or day 7 after plating. Expression of the α-actin gene in rat FSCs increases with time in culture

were most conspicous in zone 3 of Rappoport and extended in several lobules up to the peripheral zone 1. Repeated injection with CCl_4 resulted in liver fibrosis displaying septa connecting portal tracts and central veins. Focal cirrhotic transformation could also be observed.

b) Vimentin, desmin and α-SM-actin in rat liver

To study whether the results obtained in vitro could be reproduced in vivo, sections of normal, as well as CCl_4-damaged livers were fixed and stained with monoclonal antibodies against vimentin, desmin and α-SM-actin. In normal rat liver, strong vimentin staining was detected in the cells of the portal fields and in cells situated along the sinusoids (Fig. 4B). Desmin was detected in the sinusoids in a fiber-like pattern and weak staining was found in the cells of the walls of hepatic blood vessels (Fig. 4C).

No α-SM-actin staining was present in the parenchyma (sinusoids) of normal liver, but cells of the blood vessels were strongly stained (Fig. 4D).

Two days after CCl_4-administration, very strong staining with the anti-vimentin antibody was detected in the areas of necrosis and of mononuclear cell infiltration and in the liver parenchyma (Fig. 4F). In zone 3 (the area of hepatocellular necrosis and inflammatory infiltration) numerous strongly desmin-positive cells were detected among the infiltrating cells. Desmin staining was clearly detectable in the areas of inflammation and also in the liver parenchyma (Fig. 4G). Stellate cells (FSCs) could be identified (see insert). The results obtained with the anti-α-SM-actin antibody (Fig. 4H) were slightly different in that the number of α-SM-actin-positive cells present in zone 3 was clearly smaller than that of the desmin-positive cells, but actin staining could now be identified outside of the wall of the blood vessels. In liver sections obtained from rats treated chronically with CCl_4, numerous cells staining for vimentin (Fig. 4J) and desmin (Fig. 4K), and even more strongly for α-SM-actin (Fig. 4L), were found in the fibrous septa and around the pseudolobules. In the liver parenchyma, stronger staining for vimentin and for desmin was detectable but many fewer α-SM-actin-positive cells could be identified. This may indicate that only dividing FSCs (largely within fibrous septa) are able to express the α-SM-actin gene as observed in vitro.

However, the in vivo data do not exclude the possibility that desmin- and α-SM-actin-positive cells within the fibrous septa are derived from progenitor cells other than FSCs, e.g. smooth muscle cells from blood vessels.

Discussion

The data presented in this paper demonstrate that FSCs in the rat liver are able to express not only the vimentin and desmin but also the α-SM-actin gene in vitro and possibly also in vivo.

Expression of desmin and α-SM-actin increased during the time in culture in explanted cells. At day 7 after isolation all cells present in the culture were strongly vimentin-, desmin- and α-SM-actin-positive. The fact that FSCs express vimentin, α-SM-actin and desmin genes suggests a close similarity with the myofibroblasts found in hypertrophic scars and fibromatoses (Skalli et al. 1989) which show immunochemical features indicative of smooth muscle differentiation.

Our results obtained on freshly isolated FSCs are at variance with those published by Tasutsumi et al. (1987). These authors found that 90% of freshly isolated FSCs were positive for desmin and negative for vimentin using an indirect immunofluorescence technique. This discrepancy could be due the different staining technique and to the different antisera used.

FSCs maintained in culture for a longer period of time lose their fat droplets but remain desmin- and α-actin-positive. These cells are able to accumulate new fat droplets when cultured in the presence of retinol-palmitate (Ramadori et al., manuscript in preparation).

Expression of the α-actin gene in culture could also be demonstrated at the RNA-level and the total RNA extracted from cultured FSCs was compared with that extracted from skeletal and cardiac muscle from fibroblasts and hepatocytes. FSCs-RNA not only contains beta- and gamma-actin-specific transcripts, like fibroblasts and hepatocytes, but skeletal and cardiac muscle-RNA also contains α-actin-specific transcripts.

Although desmin is generally characteristic of smooth muscle cells, it has been shown to coexist with vimentin in baby hamster kidney (BHK-21) cells, in chick embryo fibroblasts (Gard et al. 1979) and in non-muscle fibroblastic cells from embryonic chick heart cell

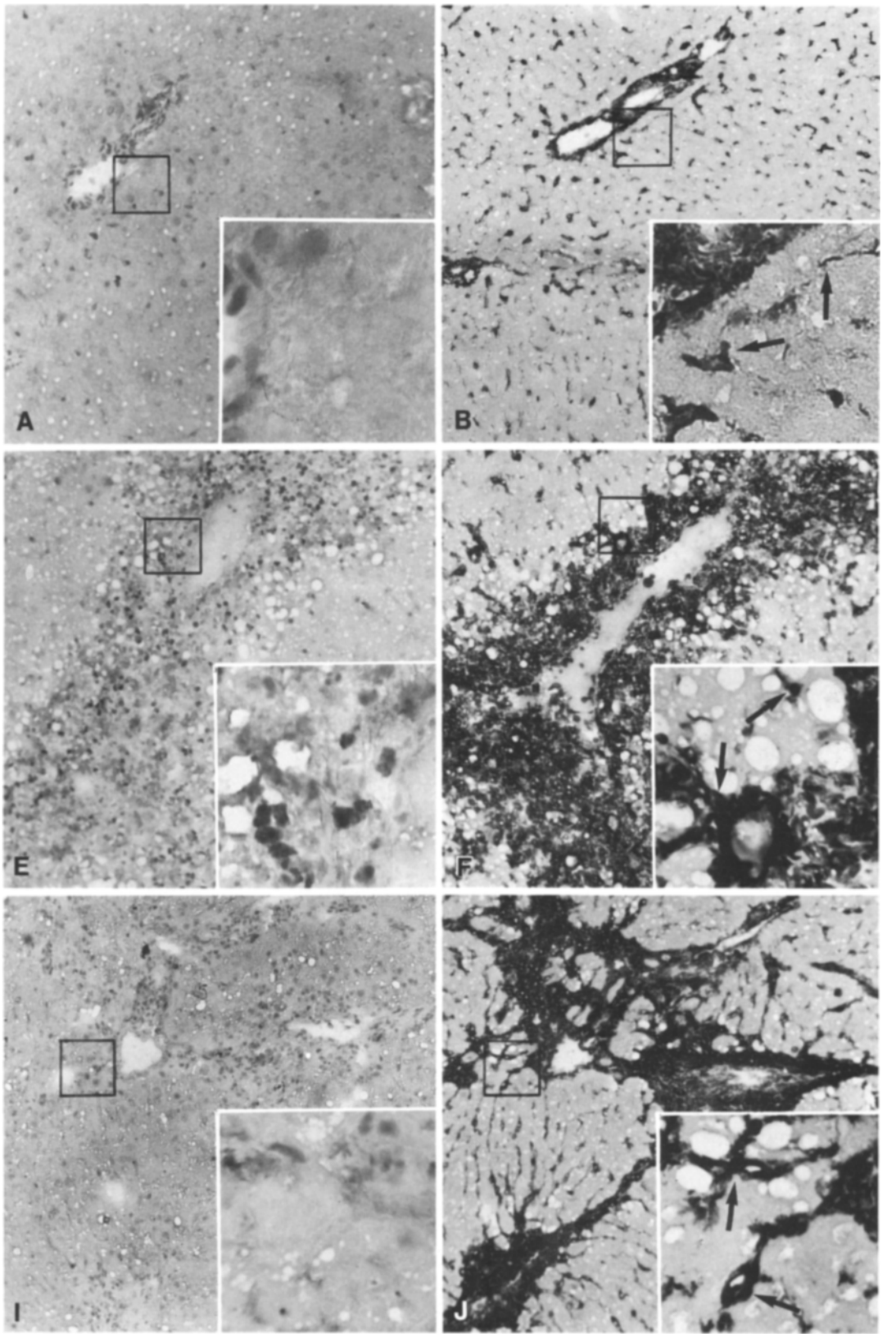

Fig. 4 A–J. In vivo expression of vimentin, desmin- and α-SM-actin-genes in normal liver **A–D**, in acutely damaged liver **E–H** or in chronically damaged liver **I–L** (see material and methods). Sequential liver sections were fixed and stained for ED 3 (**A, E, I**, negative controls), for vimentin **B, F, J**, for desmin **C, G, K** and for α-SM-actin **D, H, L**. Insert (×400) represent higher magnification of the area indicated by the square on the lower magnification picture (×100). *Arrows* indicate vimentin-, desmin-, and α-SM-actin-positive cells

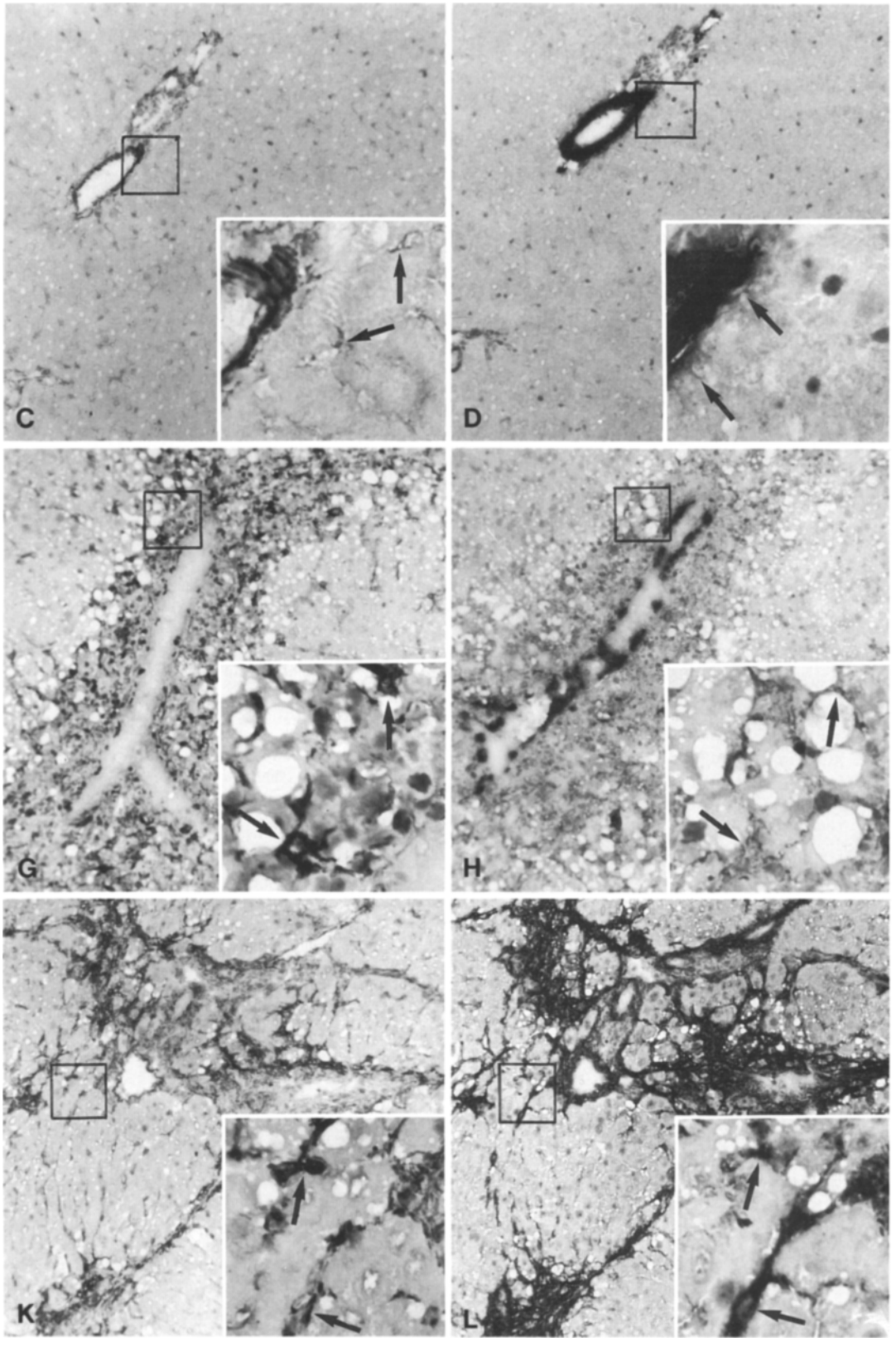

Fig. 4

cultures (Ip et al. 1983). This may mean that the presence of desmin is not sufficient to identify FSCs in culture. However, our in vitro and in vivo data confirm and further stress the hypothesis that the desmin-positive cells, found in normal liver parenchyma and in the fibrous septa of th cirrhotic rat liver, represent resident FSCs in the first case, and "activated" FSCs, developed to myofibroblasts, in the second case. At the stage when they show increased desmin- and SM-alpha-actin-gene-expression, they no longer contain fat droplets and vitamin A. Nevertheless, since specific marker for FSC-derived myofibroblasts are lacking a definitive proof for this suggestion has still to be to provided.

An increase in α-SM-actin gene-expression in "activated" FSCs in vitro, and possibly also in vivo, seems to be related to cell proliferation. In fact, α-SM-actin has been shown in the wall of blood vessels but not in the sinusoids of normal liver. α-SM-actin could represent a new marker for dividing FSCs. This would differentiate FSCs from smooth muscle cells where α-SM gene-expression seems to be downregulated during cell proliferation in vitro (Owens et al. 1986).

As it has been demonstrated that the α-SM-actin gene-expression in rat smooth muscle cells is downregulated by interferon (Hansson et al. 1989), similar experiments on FSCs are under way in our laboratory.

Recently it has been recognized that FSCs may occur in close contact with bare nerve endings (Lafon et al. 1989); the presence of contractile myofibrils suggests that these cells, which possess long projections lying underneath endothelial cells and between hepatocytes (Wake et al. 1988), could play a role in the hemodynamic regulation of sinusoidal blood flow.

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