Avidin is a major $[35\text{S}]$methionine-labeled protein induced by bacterial lipopolysaccharide (LPS) and interleukin 6 (IL-6) in cultured chick embryo myoblasts and chondrocytes. It was identified by N-terminal sequencing of the protein purified from conditioned culture medium of LPS-stimulated myoblasts. In addition, avidin was secreted by unstimulated myoblasts and chondrocytes during in vitro differentiation; maximal expression being observed in differentiated myofibers and hypertrophic chondrocytes. In developing chick embryos, immunohistochemistry revealed avidin in skeletal muscles and growth plate hypertrophic cartilage. Avidin was secreted into culture as a biologically active tetramer. Exogenous avidin added to the medium of proliferating chondrocytes progressively inhibited cell proliferation, whereas addition of avidin to differentiating chondrocytes in suspension allowed full cell differentiation. No toxic effects for the cells were observed in both culture conditions. Western blots of samples from cytosolic extracts using alkaline-phosphatase-conjugated streptavidin showed three biotin-containing proteins. Acetyl-CoA carboxylase was identified by specific antibodies. Based on these data, we propose that avidin binds extracellular biotin and regulates cell proliferation by interfering with fatty acid biosynthesis during terminal cell differentiation and/or in response to inflammatory stimuli.

Key words: Acute phase response, Inflammation, Biotin, Chondrogenesis, Myogenesis
The cells were plated in adherent conditions at a concentration of 10^5 cells/3 cm dish in Coon’s modified F12 culture medium containing 10% FCS. Ascorbic acid was added after 2 days at a concentration of 0.1 mg/ml.

Cultured myoblasts were obtained from limbs of 11-12-day-old chick embryos, as previously described (Ferrari et al., 1990). Briefly, isolated tissues were digested in 0.05% trypsin (Gibco BRL Inc., Grand Island, NY, USA) in phosphate buffered saline (PBS) at 37°C for 10-20 minutes. After proteolytic digestion, tissues were fragmented by repeated pipetting, debris were removed by filtration through a sterile nylon mesh, and cells were collected by centrifugation. The cell suspension was first plated in standard tissue culture dishes for 30 minutes to reduce the number of contaminating fibroblasts, which adhere more rapidly to the plastic, and then replated on collagen coated dishes. The culture was grown in Dulbecco’s minimum essential medium supplemented with 15% horse serum and 5% chick embryo extract.

For both chondrocytes and myoblasts, where indicated, endotoxin LPS from *Escherichia coli*, and IL-6 (Genzyme, Cambridge, MA, USA) were added for 16 hours at a concentration of 10 μg/ml and 1000 units/ml, respectively. Treatments with inflammatory agents were performed in serum-free medium.

Serum-free medium cultures of dedifferentiated chondrocytes were performed as described previously (Quarto et al., 1997). Briefly, dedifferentiated chondrocytes, derived from primary cultures grown in serum, were plated in anchorage-dependent conditions at a density of 1.2x10^3/3 cm diameter dish in the presence of serum, for 24 hours. The medium was changed and the cells kept for 48 hours in the absence of serum and then grown in serum-free medium in the presence of insulin (500 ng/ml) and fibroblast growth factor 2 (FGF2) (10 ng/ml; Austral Biological, San Ramon, CA, USA).

Serum-free medium cultures of differentiating chondrocytes were performed by plating dedifferentiated chondrocytes (5x10^3/cm^3 diameter dish) on agarose-coated dishes in the presence of insulin (500 ng/ml), T3 (10^-9 M) and dexamethasone (10^-7 M). When indicated, avidin was added to the culture medium at a concentration of 100 μg/ml, 20-fold the concentration of biotin present in the cell culture medium (LPS, insulin, T3, dexamethasone and avidin were from Sigma Chemical Co. Ltd, St Louis, MO, USA).

Cell growth curve
Dedifferentiated chondrocytes were grown in serum-free medium, as described above. Two parallel cultures were established: a control culture and a culture supplemented with avidin (100 μg/ml). After 5 days, both subconfluent cultures were trypsinized, cells were washed with PBS containing protease inhibitors (aprotinin 2.5 mg/ml, leupeptin 100 μM, PMSF 1 mM) and plated at a concentration of 4x10^3 in each well of a 24-well plate (1.2 cm diameter). The thiazolyl blue (MTT; Sigma) method was used (Denizot and Lang, 1986). At 2, 7 and 12 day culture, medium was removed and replaced with 0.5 ml of fresh serum-free medium supplemented with 25 μl MTT stock solution (5 mg/ml). After a 4 hour incubation, the medium was collected and the converted dye was solubilized with 1 ml absolute ethanol. Dye absorbance was measured at 570 nm with background subtraction at 670 nm. Cell number were determined based on a standard curve.

Cell culture labeling, polyacrylamide gel electrophoresis and immunoprecipitation
Culture dishes were washed with PBS, incubated at 37°C for 2 hours with methionine-free medium and labeled for 2 hours with [35S]methionine (100 μCi/ml), as previously described (Descalzi Cancedda et al., 1988). Chondrocyte labeling was always performed in the presence of ascorbic acid (100 μg/ml).

Aliquots of culture media were loaded on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis, as described (Gentili et al., 1998), in reducing conditions unless otherwise indicated. Polyacrylamide gel concentration was 10%.
Immunoprecipitation was performed as described (Descalzi Cancedda et al., 1988). Briefly, aliquots from labeled culture media were incubated with pre-immune rabbit serum for 1 hour at 4°C. Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) was added to each sample and the incubation continued for a further 30 minutes. After removing the Pansorbin by centrifugation, proteins were incubated overnight with rabbit anti-chicken avidin antiserum (Sigma). Pansorbin was added for 30 minutes, the immunocomplexes were washed three times in PBS containing 1% Triton X-100, 0.1% SDS and 5 mM EDTA, dissolved in the electrophoresis sample buffer and analyzed by SDS-PAGE.

To identify pepsin-resistant proteins, 35S-labeled proteins secreted in the culture medium were dialysed against 0.5 M CH3COOH and digested overnight with pepsin (0.1 mg/ml). Aliquots were analysed by SDS-PAGE.

**Protein purification, blotting and sequencing**

Serum-free cell culture medium from LPS-stimulated myoblasts was concentrated in a Centriprep-10 (Amicon Inc., Beverly, MA, USA). Radioactive methionine-labeled medium from the same cells was added as a tracer and, after dialysis against PBS, the concentrated medium was loaded onto a Superdex-75 FPLC column (1×30 cm; Amersham Pharmacia Biotech, Amersham Italia, Milan, Italy) equilibrated with PBS. The flow rate was 0.5 ml/minute; fractions of 0.5 ml were collected and 0.1 ml of each fraction was analyzed by SDS-PAGE and revealed by autoradiography of the dried gel.

Fractions 18-19 from the Superdex 75 column were collected, and protein was precipitated with 10% trichloroacetic acid and run onto a 15% polyacrylamide gel electrophoresis. After electrophoresis the gel was electroblotted in CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer onto a ProBlott membrane (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Milan, Italy). N-terminal sequencing was performed on a protein sequencer model Procise 492 (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA).

**Western blot analysis**

For avidin identification in conditioned culture media, medium aliquots were loaded on a 15% SDS-PAGE. Electrophoresis was performed in reducing conditions. After electrophoresis the gel was blotted to a BA85 nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany), according to a previously described procedure (Towbin et al., 1979). The blot was saturated for 16 hours with 5% nonfat cows milk in TTBS buffer (20 mM Tris HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20), washed several times with TTBS and incubated for 2 hours at room temperature with a rabbit anti-chicken avidin antiserum (Sigma). After washing, the detection was performed by a conjugated alkaline phosphatase anti-rabbit IgG (Boehringer Mannheim GmbH, Mannheim, Germany), using the alkaline

![Fig. 1. Proteins released by cultured chick embryo myoblasts (A) and chondrocytes (B) stimulated by inflammatory agents. Cells were stimulated by the bacterial endotoxin lipopolysaccharide (LPS) or interleukin 6 (IL-6) for 16 hours before labeling with [35 S]methionine. Labeled proteins released into the media were analysed on a 15% SDS-PAGE. (A, B) Lane 1, control; lane 2, culture-treated with LPS; lane 3, culture-treated with IL-6. The number on the left refers to molecular mass markers (kDa).](image)

![Fig. 2. Purification of the 15.5 kDa protein. The protein was purified from myoblast-conditioned medium by FPLC on a Superdex 75 column (inset), followed by SDS-PAGE of each chromatographic fraction (inset). Numbers at the top indicate the fraction numbers; T indicates total samples applied to the column; numbers on the left refer to molecular mass markers (kDa); arrows indicate the 15.5 kDa protein.](image)
phosphatase solution BCIP/NBT (ICN Biomedicals, Costa Mesa, CA, USA) as a substrate. The detection with ECL was performed using a horseradish peroxidase linked to anti-rabbit IgG (Amersham Pharmacia Biotech), using the ECL reagents from Amersham Pharmacia Biotech as the substrate.

For detection of biotin-enzymes in cell extracts, samples were boiled and loaded on 7.5% SDS-PAGE. Blotting and milk saturation was performed as above. Blots were then incubated with alkaline-phosphatase-conjugated streptavidin (1:500; Jackson). Identification of avidin as one of the major products of conditioned medium; lane 2, immunoprecipitated avidin; double the amount of counts were used for immunoprecipitation with respect to control. Numbers on the left refer to molecular mass markers (kDa).

**Immunohistochemistry**

Tissue biopsies were fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin. Serial sections (5 μm) were dewaxed and rehydrated in xylene (1:300). After additional washing of the sections with PBS and 50 mM Na acetate, pH 5, the peroxidase activity was visualized by enzymatic modification of the 3-amino-9-ethylcarbazole substratum (0.4% 3-amino-9-ethylcarbazole in dimethylformamide, 50 mM Na acetate, pH 5, 30% H2O2, 100:900:1) during a 15 minute incubation in the dark at room temperature. Sections were then counterstained with 0.02 M Seromed Biochrom KG, Berlin, Germany) containing protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM PMSF). Cells were broken by glass potter in ice and centrifuged at 50,000 g for 40 minutes. Cytosol extracts were aliquoted and frozen at −80°C. Alkaline-phosphatase solution BCIP/NBT (ICN Biomedicals, Costa Mesa, CA, USA) as a substrate. The detection with ECL was performed using a horseradish peroxidase linked to anti-rabbit IgG (Amersham Pharmacia Biotech), using the ECL reagents from Amersham Pharmacia Biotech as the substrate.

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**RESULTS**

**Identification of avidin as one of the major products secreted by cultured myoblasts and chondrocytes in response to inflammatory agents**

Chick embryo myoblasts were expanded in culture, stimulated
Avidin in development and inflammation

with the bacterial endotoxin LPS or IL-6 and labeled with $^{[35}S]$methionine. Proteins released into the culture medium by the stimulated and the control unstimulated cells were analysed by SDS-PAGE (Fig. 1A). As previously reported (Cermelli et al., 2000), we observed a strong enhancement in the secretion of several low molecular mass proteins (<25 kDa).

Enhancement in the secretion of the same proteins was observed when labeled proteins released by cultured stimulated chondrocytes were compared with proteins released by unstimulated chondrocytes (Fig. 1B). The identification of the 21 kDa protein as Ex-FABP has already been reported (Descalzi Cancedda et al., 1996).

The 15.5 kDa protein was purified by FPLC on a Superdex 75 column and SDS-PAGE from the conditioned medium of LPS-stimulated myoblasts (Fig. 2). The blotted band was directly loaded onto the sequencer, and the N-terminal sequence was determined up to residue 22. The sequence obtained was: ARKXSLTGKWTDLGSXTMTIA. No identifiable residues were detected at positions 4 and 17.

A search in SWISS-PROT identified the protein as the mature form of chicken egg avidin (accession number P02701), which has a cysteine residue involved in a disulfide bridge at position 4, and a glycosylated asparagine residue at position 17. The identity with chicken egg avidin and the non-identity with the product of any of the avidin-related genes (AVR) was confirmed by the inspection of the few variable positions among the different sequences (Fig. 3).

The nature of the 15.5 kDa protein present in the culture medium of both myoblasts and chondrocytes was confirmed by immunoprecipitation of the protein with commercial antibodies raised against the chick avidin from the egg white (Fig. 4).

The expression of avidin is enhanced during terminal differentiation of myoblasts and chondrocytes in the absence of inflammatory stimuli

We have previously reported that Ex-FABP secretion was ‘physiologically’ increased during differentiation of both myoblasts and chondrocytes (Cermelli et al., 2000). In view of this finding, we have analysed proteins released into the culture medium by myoblasts and chondrocytes at several differentiation stages (Fig. 5). As in the case of Ex-FABP, we have observed that the amount of avidin released into the medium progressively increased during myoblast culture in parallel with the increased number of detectable myofibers (Fig. 5A). Similarly, in chondrocyte cultures avidin secretion increased with the progressive increase of hypertrophic chondrocytes (Fig. 5B). The presence of avidin in the culture medium conditioned by both myoblasts and chondrocytes at a late stage of differentiation was confirmed by western blot analysis. It is of note that the avidin present in the conditioned media had the same molecular mass as chicken egg avidin.

Presence of avidin was observed in terminally differentiating myoblasts and hypertrophic chondrocytes in vivo. In sections of a 10-day-old chick embryo hindlimb, antibodies raised against the egg white avidin localized the protein in the forming skeletal myofibers and in the hypertrophic cartilage region of the tibia growth plate (Fig. 6).

Avidin secreted by cultured cells is a stable tetramer with biotin binding activity

A concentrated serum-free cell culture medium from LPS-stimulated hypertrophic chondrocytes was extensively dialysed to remove unbound biotin and divided into two aliquots. The two aliquots were incubated for 20 minutes with $^{[14}C]$biotin, one at 25°C and the other at 90°C, dialysed against PBS and subsequently run on a gel filtration FPLC column. Fig. 7 shows radioactivity bound by avidin during the incubation at 25°C or 90°C. After incubation at 25°C, a small amount of radioactivity remained in the sample after the dialysis and all this radioactivity eluted in a single peak from the column. The radioactive peak coeluted with the secreted avidin identified in the column fractions by western blot analysis (Fig. 7, inset). It should be
noted that after incubation in the presence of radioactive biotin at both low and high temperatures, avidin always eluted in the same chromatography fractions. Coelution with the radioactive biotin was observed only after incubation at 90°C. As expected, this indicates that, owing to the high affinity binding, exchange of non-radioactive biotin (presumably derived from culture medium) with radioactive biotin can occur only at high temperatures. The calculated molecular mass of avidin on the calibrated column was 62 kDa, indicating a tetrameric quaternary structure. Commercial chicken egg avidin eluted in the same fractions as avidin secreted by cultured cells (not shown). Avidin secreted by cells in culture is stable; it does not alter its quaternary structure at 90°C.

Search for biotin-enzymes in cell extracts

Given the exceptionally strong affinity of avidin for biotin, one can assume that, in the presence of a high concentration of extracellular avidin, the vitamin is sequestered in the extracellular microenvironment. Thus intracellular biotin could become limiting for the cell metabolism. In an attempt to investigate metabolic pathways potentially affected, we performed experiments aimed at the identification of intracellular biotinylated proteins. Biotinylated enzymes play a key role in fatty acid and lipid metabolism. Western blot analysis of cytosol extracts of both differentiated chondrocytes (Fig. 8A, lane 1) and myoblasts (lane 2), revealed with streptavidin alkaline phosphatase, showed three bands with an apparent molecular mass of >200 kDa, 130 kDa and 75 kDa. Based on their apparent molecular mass and the information existing in the literature (Thampy, 1989), the three proteins were tentatively identified from top to bottom as: acetyl-CoA carboxylase, pyruvate carboxylase and the β subunit of the propionyl-CoA carboxylase. Acetyl-CoA carboxylase is a key enzyme for de novo fatty acid biosynthesis. It is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, a substrate for fatty acid synthase. Antibodies that recognize this enzyme were used for a western blot analysis and the protein with the higher electrophoretic migration was definitely identified as acetyl-CoA carboxylase in myoblasts (Fig. 8B) and chondrocytes (not shown).

Avidin prevents proliferation but not differentiation of chondrocytes in culture

We have reported that dedifferentiated chondrocytes can be maintained in a proliferating status in serum-free conditions by culturing the cells in the presence of insulin and FGF-2 (Quarto et al., 1997). A possible interference of exogenously added avidin with chondrocyte proliferation and differentiation was investigated. Parallel cultures were established either with or without the further supplement of egg white avidin to the medium. The protein was added at a concentration 20-fold that of biotin in the medium. Dedifferentiated chondrocytes cultured in the presence and in the absence of avidin had initially identical growth rates (not shown), but when the subconfluent cultures were passaged, after approximately 2-3 cell doublings, we observed a marked decrease in the proliferation rate of the cells cultured in the presence of avidin (Fig. 9A). The cell growth arrest persisted for several days; after only about 2 weeks from replating, some resumption of proliferation was observed (not shown).
These data suggest that exogenously added avidin is not toxic for the cells and that avidin presumably acts by sequestering biotin from the medium. Control dedifferentiated chondrocytes cultured without addition of avidin continued to proliferate throughout the whole culture. Interestingly, cell culture labeling at 12 days shows a strong induction of Ex-FABP in avidin-treated cells (Fig. 9B).

Dedifferentiated chondrocytes transferred into suspension culture in serum-free medium undergo differentiation when cultured in the presence of thyroid hormone T3, insulin and dexametasone (Quarto et al., 1992). When we have cultured dedifferentiated chondrocytes in these conditions, in the presence and in the absence of chicken egg avidin, we have observed that avidin did not interfere with cell differentiation and that in both cultures, cells underwent maturation to hypertrophic chondrocytes synthesizing and releasing the cartilage-specific type II and type X collagens (Fig. 9C). By western blot analysis, type X collagen was detected in cell extracts of chondrocytes differentiating in suspension both in the absence and in the presence of avidin, whereas it was absent in dedifferentiated chondrocytes (Fig. 9D).

DISCUSSION

In the existing literature, avidin is considered as a component of egg albumen that might act as a strong antibacterial agent. Avidin is produced in the oviduct, but it is induced in other tissues following injection of inflammatory agents. If subjected to viral transformation or cell damage, chicken fibroblasts in vitro express the protein.

In this manuscript we have reported expression of avidin both in growth plate hypertrophic cartilage and in developing muscle of chick embryos. Avidin secreted by cultured chondrocytes and myoblasts presented a molecular mass of 62 kDa (determined by gel filtration), as expected for a tetrameric quaternary structure. Secreted avidin showed high thermal stability and heat-induced biotin exchange. Western blot analysis of myoblast and chondrocyte extracts using avidin phosphatase identified three biotin proteins of molecular mass >200, 130 and 75 kDa, tentatively identified as acetyl-CoA carboxylase, palmitoyl CoA carboxylase and piruvate carboxylase. The identity of the acetyl-CoA carboxylase was confirmed by a western blot analysis with antibodies directed against the rat protein and crossreacting with the chicken protein. Chicken egg avidin added to culture medium of proliferating dedifferentiated chondrocytes inhibited cell proliferation, whereas the addition of avidin to the culture medium of differentiating chondrocytes did not interfere with their differentiation.

Fig. 8. Detection of biotin enzymes in myoblast and chondrocyte extracts. For detection of biotin enzymes, blots were incubated with alkaline-phosphatase-conjugated streptavidin; alkaline phosphatase activity was revealed with the BCIP/NBT solution (A). Lane 1, chondrocytes; lane 2, myoblasts. Acetyl-CoA carboxylase identification was performed in myoblasts using polyclonal antibodies against acetyl-CoA carboxylase; detection was performed by a conjugated peroxydase anti-sheep IgG, using ECL reagents as substrate (B). Numbers on the left refer to molecular mass markers (kDa).
Avidin is characterized by a high affinity for biotin; in particular, extracellular avidin could sequestrate all biotin in the extracellular microenvironment per se and to transport them into the cells (unpublished data), specifically bind fatty acids (Descalzi Cancedda et al., 1996) and to transport them into the cells (unpublished data), suggests that Ex-FABP acts as scavenger for free fatty acids released by the cells. This latter event might be used by cells to compensate in part for the effects of reduced fatty acid formation.

It is of note that in cultures of 3T3-L1 murine preadipocytes, a mediator substance released by macrophages in response to LPS causes a decrease in the synthesis and activities of enzymes involved in de novo fatty acid biosynthesis (Pekala et al., 1983). This was contemporary to a dramatic decrease in the lipoprotein lipase activity. In general, lipoprotein lipase is used by cells as a way to hydrolyze extracellular triacylglycerol to partial glycerides and free fatty acids. The liberated fatty acids are then taken up by the cells and plays a role by retarding fatty acid biosynthesis and, subsequently, cell proliferation. Overall, we can hypothesize that, as a consequence of the decreased availability of the biotin necessary for activation of fatty acid synthesis de novo, in the terminally differentiating and apoptotic cells the lipid catabolic pathway could prevail on the anabolic pathway.

We have observed that avidin supplements added to culture medium result in Ex-FABP induction. Therefore, in chicken differentiating myoblasts and hypertrophic chondrocytes the avidin-dependent inhibition of enzymes involved in de novo fatty acid biosynthesis might be contemporary to the induction of Ex-FABP synthesis. The capability of Ex-FABP to specifically bind fatty acids (Descalzi Cancedda et al., 1996) and to transport them into the cells (unpublished data), suggests that Ex-FABP acts as scavenger for free fatty acids released by the cells. This latter event might be used by cells to compensate in part for the effects of reduced fatty acid formation.
Therefore it appears that in this cell system there is a coordinated regulation of fatty acid synthesis de novo and fatty acid transport from the outside to the inside of the cells.

In conclusion, despite the fact that avidin is a well known protein, its function is still largely obscure. We believe that our data give a major contribution to the elucidation of this protein function. In fact, we have demonstrated that avidin is physiologically expressed by chondrocytes and myoblasts at a late differentiation stage both in culture and in vivo. Because avidin is a biotin-binding protein that can inhibit fatty acid synthesis, this finding deserves some attention. We believe that because avidin binds extracellular biotin, it cannot be without effect at the site of its synthesis and secretion. Fatty acid biosynthesis is an important process for membrane building and signal transduction during cell proliferation. We related the protein synthesis and secretion by terminally differentiated cells to the cell proliferation arrest. Our data suggest that avidin is made by cells at a late differentiation stage when they need to arrest proliferation but continue the differentiation process.

In addition, we have shown that, in vivo, avidin is made by the same terminally differentiated cells; therefore it is likely that avidin can bind extracellular biotin and regulate cell proliferation in the tissues, as demonstrated in vitro.

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