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Hyaluronectin Level in Heart Cell Cultures in Presence of Food Antioxidants

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any University.

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ABBREVIATIONS

ECM	: Extracellular matrix
GAG	: Glycosaminoglycans
HABP	: Hyaluronic acid binding protein
ELISA	: Enzyme linked immunosorbent assay
HA	: Hyaluronic acid
PG	: Proteoglycans
SGP	: Structural glycoproteins
Mw	: Molecular weight
IgG	: Immunoglobulin type G
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
NBT	: Nitroblue tetrazolium chloride
BCIP	: Bromo-4-chloro-3-indolephosphate-toludine salt
pNPP	: p-Nitrophenyl phosphate
TEMED	: N, N, N', N'- tetra methylene diamine
TRIS	: Tris (hydroxymethyl) amino methane
BSA	: Bovine serum albumin
BHA	: Butylated hydroxy anisole
BHT	: Butylated hydroxy toluene
FCS	: Fetal calf serum
HS	: Horse serum

AIM AND SCOPE

Extracellular matrix (ECM) constitutes an intricate network of macromolecules which largely fills the extracellular space. The ECM is a structurally stable material that lies under epithelia and surrounds connective tissue that denotes the material which joins up the other three primary tissues i.e., epithelia, muscle and nervous.

During the last decade, knowledge about the descriptive and molecular biology of ECM has increased exponentially. Cellular responses to contact with extracellular matrix are varied and include adhesion, spreading, differentiation, migration, invasion and remodeling. The extracellular matrix macromolecules of different animal species from invertebrates to human can be arranged in four major categories or families; collagens, elastins, proteoglycans (PGS) and structural glycoproteins (SGP). The first two families form the fibrous scaffolding of connective tissues, the PGS and SGPs take part in filling of the interstices and the interfaces between cells and ECM.

Collagen a major structural component of tissues, comprises an impressive 30% or more of the protein of adult animals. Fourteen different collagen types have been recorded till now (Labat _et al_ 1990). The classical triple helix represents a quantitatively more or less important but functionally essential part of these molecules. Elastin, physiologically speaking is the youngest matrix molecule appearing in its present form only in

vertebrates. It is a product of a single gene. Elastin is associated with microfibrillar structures. It is highly hydrophobic, non-glycosylated protein.

Proteoglycans are glycosaminoglycans found covalently attached to a protein backbone, except for hyaluronan or hyaluronic acid. The carbohydrate chains of proteoglycans are among the well characterized ECM components containing repeating units of hexosamine and uronic acids (D-glucosamine, D-galactosamine, D-glucuronic acid, L-iduronic acid). Other than carbohydrate rich cartilage proteoglycans, there are others with much lower carbohydrate to protein ratio such as dermatan-sulphates of the skin, heparin-sulphate and chondroitin-sulphate proteoglycans on the cell membranes and basement membranes.

Structural glycoproteins are characterized by the presence of asparagine or serine linked oligosaccharides. They are built mostly of globular domains linked by more flexible regions. Well characterized examples of structural glycoproteins are fibronectin, laminin, chondronectin and elastonectin.

Among the different glycosaminoglycans, hyaluronic acid has an important role in several functions of the cell, especially in the early stages of morphogenesis. Differential expression of HA is evident during cell differentiation. There is direct correlation between the presence of HA in extracellular spaces and cell migration (Toole et al, 1984,1987).

The ability of environmental cues to alter characteristic of myogenesis is now well established (Konigsberg, 1987; Ahrens et

al 1979; Chiquet et al 1981). Myoblasts require collagen (or gelatin) to attach to tissue culture substrata, but their differentiation is blocked by HA (Elson and Engwall, 1980; Kujawa et al, 1986). Even though myoblasts synthesize and secrete HA and chondroitin-sulphate, the hypothesis has been raised that myoblasts shed cell surface GAG prior to fusion. The effect of HA on myogenesis stands in opposition to its demonstrated ability to enhance chondrogenesis of competent mesenchyme (Kujawa and Caplan, 1986).

Knudson and Toole (1987) have shown the mode of interaction of hyaluronate with the cell surface, changed at the onset of mesodermal cell condensation prior to differentiation of cartilage and muscle. The hyaluronate binding sites appeared on the cells and continued to be present on differentiated chondrocytes but not on myotubes.

Several lines of evidences from us and others confirmed the existence of a family of hyaluronic acid binding proteins (HABP or hyaluronectins), which seems to be regulating the levels of HA in ECM of transformed cells, normal cells and embryonic cells. Delpech et al (1982) first reported the presence of a HABP from human nodes of Ranvier. From our laboratory in 1985, we reported the presence of a naturally occurring protein which binds to HA and purified this protein from normal rat brain and liver (D'Souza and Datta, 1986a). The affinity of HABP (Hyaluronectin) to different glycosaminoglycans was further examined (D'Souza and Datta 1986b), and its specificity towards HA only was confirmed.

Furthermore, by amino acid analysis our laboratory confirmed that the HABP is distinct from fibronectin, laminin and gelatin-binding proteins which are also known to bind HA. Gupta et al (1991) have purified and partially characterized rat kidney HABP and have shown its localization on cell surface. Their observation on the interaction of HABP with fibronectin, laminin and collagen type IV besides HA suggests its possible role in structural organization under normal and pathological conditions.

In the present investigation, our interest was to elucidate the role of HABP, a cell surface associated glycoprotein, during cell differentiation under normal condition and in the presence of an environmental factor e.g., butylated hydroxy anisole (BHA) which is a commonly used food antioxidant. In our current study, we have used neonatal rat heart myoblasts as it provides an excellent model for muscle differentiation. Cultured heart cells offer some advantages over other preparations like, the direct effect of drugs or other chemical agents could be studied without equivocation since the cells are denervated. Further, pure myocardial cell cultures can be prepared, thus, studies can be made without contamination by other cell types present in the myocardium.

Many a scientist have already shown the role of other major glycoproteins such as fibronectin, laminin and chondronectin during cell differentiation. Our studies on the involvement of HABP, a novel ECM protein in myoblast differentiation may unravel the regulatory role of ECM components during the process of myogenesis.

INTRODUCTION

The biology of the extracellular matrix is an interesting field. The extracellular matrix is structurally stable material that lies under epithelia and surrounds connective tissues. Since, the cells and their extracellular matrices form a biochemical continuum, cellular properties are continuously related and dependent on the composition and organization of the matrix. Much of the volume of tissue in animals consists of spaces between cells, and the essential entities do lie outside the plasma membrane. A variety of proteins that are attached to the outer surface of the animal cells participate in formation of specific contacts and functions between cells and as a consequence imparts strength and rigidity to the multicellular tissues.

Constituents of extra cellular matrix

Important constituents of the connective tissue extracellular matrices are collagen, proteoglycans and structural glycoproteins.

Collagen

Collagen is the principal component of ECM which endures the mechanical stability of connective tissues. Collagen constitutes a family of large molecular weight proteins. The molecule gives support and tensile strength to the cuticle of annelid worms and skin, bone, tendons and cornea of vertebrates. It is known that there are atleast five different collagens within an individual organism.

Proteoglycans

Proteoglycans, the important constituent of ECM are diverse group of heterogeneous macromolecules. These are most abundant in the ECM of connective tissues and contain core proteins to which one or more glycosaminoglycan side chains are covalently attached.

Structural glycoproteins

They are defined as glycoproteins which are extractable with urea or/and mercaptoethanol from TCA insoluble residues of fibrous stroma of different connective tissues and present in the ECM. Several of them such as fibronectin, laminin, integrin, thrombospondin, tanasin, undulin etc. are shown to play an important role in ECM.

Apart from these well characterized proteins of the ECM, in the recent years significant advances have been made in characterizing and understanding the functions of hyaluronic acid binding proteins in cell behavior. Toole (1990) has coined the term hyaladherins for this new family of extracellular matrix proteins which show high affinity for hyaluronic acid.

ECM plays an active role in morphogenesis and cytodifferentiation. Differentiation can be viewed as a series of morphogenetic events, a series of integrated interactions among cell populations and the environment each creates around itself, such that sum of these interactions leads to an ordered acquisition of tissue specific characteristics.

The model system is cardiac muscle, which is extremely heterogeneous as to cell type. The two major cell types of this soft tissue, fibroblasts and myoblasts are closely interspersed. It is difficult to pinpoint the precise time of onset of differentiation of muscle but once initiated it proceeds very asynchronously.

As an experimental system, muscle cell culture offers distinct advantages that are unmatched in the intact embryo. By exploiting the differential adhesive properties of myoblasts, we can selectively detach large number of these myogenic cells from primary cultures relatively free of other contaminating cell types. Moreover, the use of cell culture allows the investigator to rigidly control the environment and to employ a spectrum of experimental interventions, difficult if not impossible to achieve in the organisms. One can manipulate culture conditions to impose a high degree of synchrony of differentiation and thereby an extremely reproducible pattern of development.

Cardiogenesis can be separated into two phases; pre-tubular phase of cell migration that leads to the formation of heart tube and tubular heart phase of complex morphogenic changes that leads to the definitive fetal heart. During the pre-tubular heart phase of cardiogenesis, the cellular primordia of the myocardium and endocardium are formed. In addition a well organized extracellular matrix is produced.

The role of ECM components in myogenesis

Fibronectin

Fibronectin is a 500 kDa glycoprotein consisting of two similar 250 kDa subunits held together at their carboxyl termini by a pair of disulphide bonds. The subunits are flexible and can be found fully extended to form a "V" shaped molecule (Engel *et al*, 1981; Erikson *et al*, 1981; Odermatt *et al*,1982) or folded into a compact globular molecule (Williams *et al*, 1983).

Fibronectin exists in two principal forms, a soluble form and insoluble form. Soluble protomeric fibronectin is found at high concentration in plasma and other body fluids and is synthesized and secreted *in vitro* by many cell types. Insoluble fibronectin consists of high molecular weight disulphide bonded multimers of fibronectin and is found in connective tissues, basement membrane and extracellular matrices (Mosher, 1980, 1984; Hynes, 1981; Mosher and McKeown-Longo, 1985; Hedman and Vaheri, 1989). The important biological activities of fibronectin are cell-substratum adhesion, cell motility, cell-cell adhesion, maintenance of normal cell morphology, embryonic differentiation and non-immune opsonization by macrophages.

Fibronectin is a major extracellular tissue component of muscle (Stenman and Vaheri, 1978 ; Walsh *et al* , 1981). The exact function of fibronectin in muscle is not known. Several studies on muscle cell surface in tissue culture have yielded data on the changes in the level of fibronectin during myogenesis. Cultured myoblasts have surface fibronectin as the culture becomes denser

the amount of fibrillar matrix of fibronectin increases (Hynes _et al_, 1976; Chen, 1977; Walsh _et al_, 1981). As myotubes differentiate they lack fibrillar surface fibronectin (Chen, 1977; Furcht _et al_, 1978; Walsh _et al_, 1981) and there is a drop in the amount of fibronectin at the time of fusion (Chen, 1977; Podleski _et al_, 1979). The addition of exogenous fibronectin to rat myoblast cultures inhibited fusion (Podleski _et al_, 1979) and fibronectin was also found to prevent the fusion block in the tunicamycin treated myoblast cultures (Chung and Kang, 1987). It has been claimed that fibronectin synthesis, as a fraction of total protein synthesis, remains constant before and after myoblast fusion (Gardener and Famberough, 1983). Chung and Kang (1990) observed that the amount of fibronectin in the cell surface pool as measured by immunoblotting decreases during myogenesis. This decrease or loss of fibronectin during myoblast fusion is believed to be closely correlated with the alteration of fibronectin receptors and with the fusion of myoblasts. Growing myotubes in myoblast cultures accumulate laminin and type IV collagen on their surface whereas fibronectin assembles into an intracellular fibrous meshwork, not associated with the free myotubes surface (Kuhl _et al_, 1986). Compared to fibroblasts, the myoblasts and myotubes synthesize little fibronectin of their own and also accumulate little of it on their surfaces (Chiquet _et al_, 1981). Boudreu _et al_ (1991), have observed a two fold increase in fibronectin synthesis during ductus arteriosus smooth muscle cell migration. Boudreu and Rabinovitch (1991) observed a gradient of fibronectin increasing towards the lumen of the ductus arteriosus.

Chick heart tube consists of myocardium and epithelium separated by a myocardial derived basement membrane, which is an expanded cardiac jelly. Fibronectin localized in particles (0.1-0.5 μ in diameter) appears as a gradient of decreasing concentration extending from the myocardium towards the endothelium. In contrast, no particulate fibronectin staining was observed in the ventricular region. The regionally specific interaction of myocardium with endothelium is required to initiate the formation of pre-valvular mesenchyme. This interaction may be mediated by a multicomponent complex involving fibronectin and other proteins of cardiac jelly which appears as a regionally distinct particulate only in areas of endothelial differentiation (Mjaatvedt et al, 1987).

Laminin

Another important structural glycoprotein laminin is a characteristic component of basement membranes. In contrast to more generally distributed extracellular proteins such as fibronectin, laminin is generally present only in the basal lamina of a wide variety of tissues mainly in epithelial cells, endothelial cells and myotubes (Timple et al, 1979; Chung et al, 1979). Laminin is enriched with cysteine, has relatively large amount of carbohydrate (12-15%) with significant amount of sialic acid (4-6%). Laminin is composed of subunits of 200,000 and 400,000 daltons linked by disulphide bonds. A complete laminin molecule which totals approximately 1,000,000 daltons. Laminin is usually reported to function as an adhesive protein. The extent of cell type specificity of laminin compared to other attachment

proteins, is still uncertain.

Growth of skeletal muscle occurs by fusion of multinucleated myotubes with differentiated, fusion capable myoblasts. *In vitro*, growing myotubes in myoblast cultures accumulate laminin and type IV collagen on their surfaces in patches and strands as it is a first step in assembling a continuous basal lamina on mature myofibres (Kuhl *et al*, 1986). Enhanced affinity of laminin to myogenic cells as compared to fibrogenic cells of skeletal muscle has been observed. Similarly, the adhesive property of laminin in accordance with the attachment behavior of other basement membrane associated cell types have been described such as epidermal cells (Murray *et al*, 1979), epithelial cells (Terranova *et al*, 1980), certain tumor cells (Terranova *et al*, 1982) and adult cardiac myocytes (Borg *et al*, 1983, 1984). Inhibition by a monoclonal antibody of chick myoblast attachment and isolation of a laminin receptor using the antibody have provided evidences for the existence of a laminin receptor on myoblast and its role in myoblast adhesion (Neff *et al*, 1982; Horwitz *et al*, 1985). Darmon (1982) observed that cells from multidifferentiated tumors that contain muscle tissue do not differentiate in culture media supplemented with serum. In serum free medium, differentiation was limited to neuron formation, myogenesis occurred only when medium was supplemented with laminin.

Foster *et al* (1987), reported that culture of newborn rat hind limbs grown on laminin showed prolonged proliferative phase, results in exhibiting a five to six fold enrichment of myogenic

cells. They have also shown that the capacity of rat myoblasts to respond to laminin depends on the stage of development of donor animal. In culture medium which promotes differentiation of myoblasts and myotubes, laminin synthesis increased, initially accumulated as a soluble form in the medium but later as an insoluble cell-associated fraction. The increase in surface laminin paralleled that of the accumulation of insoluble laminin suggesting that the insoluble fraction represents laminin bound to the extracellular matrix at the cell surface (Olwin and Hall, 1985). MM14 myoblast, a myogenic cell line derived from normal adult skeletal muscle showed a rapid induction of outgrowth of cell process (associated with stimulation of motility), resulting in bipolar spindle shaped cells (Ocalan et al, 1988).

It has been reported that adult cardiac myoblast reveals a high affinity to type IV collagen and laminin than to type I collagen (Borg et al, 1984). The embryonic heart mesenchymal cells migrate vigorously within the three dimensional gels of laminin and the YIGSR peptide from the B 1 chain of laminin which corresponds to the active site of cell adhesion, seems to completely abolish this migratory activity. It has been shown that laminin and type IV collagen are present in the expanded basement membrane (cardiac jelly) which is invaded by mesenchymal cells during heart myogenesis (Davis et al, 1989).

The heart forming region of the early embryo is composed of splanchnic mesoderm, endoderm and associated ECM. In chick heart primordia, the basement membrane of the endoderm contains laminin and collagen type IV. The nascent basement membrane of heart

splanchnic mesoderm contains immunoreactive laminin but not type IV collagen. The prominent ECM between splanchnic mesoderm and endoderm (primitive heart ECM) contains collagen type IV, collagen type I and fibronectin but not laminin (Drake et al, 1990).

Hyaluronic acid

In addition to the structural glycoproteins, some of the GAGs present in ECM have an important role in cell differentiation, of which hyaluronic acid is a major component. Hyaluronic acid is a non-sulfated glycosaminoglycan, high molecular weight, highly anionic polysaccharide composed of 200-10,000 disaccharides of β-1,4-glucuronate-β-1,3-N-acetyl glucosamine having a molecular weight of between $1x10^5$ and $5x10^6$ daltons, usually at the higher end of this range. It constitutes a major portion of the extracellular matrix in connective tissue and is usually found as a principal GAG in the ECM of mesenchyme, neural tissues and cell free spaces (Solursh and Morris, 1977; Greenberg and Pratt, 1977).

Hyaluronic acid interacts with other extracellular or matrix components to provide stability and elasticity to the extracellular environment. Apart from its various physico-chemical properties, it also has important biological functions in tissues. It is involved in the control of morphogenesis correlated with cell migration and cell proliferation (Toole, 1981; Toole et al, 1984; Toole et al, 1987). In the embryonic limb, hyaluronate levels are high initially and then decreases

during cyto-differentiation of cartilage and muscle (Toole, 1982; Shambaugh and Elmer, 1980; Knudson and Toole, 1985).

The association of hyaluronate with the cell surface can influence the behavior of cells especially with regard to modulation of cell aggregation and cell movement. Hyaluronate binding sites have been demonstrated on the surface of liver endothelial cells (Smedsrod et al, 1984), embryonic heart fibroblasts (Turley and Torrance, 1984), embryonic myocardial and cushion cells (Bernanke and Orkin, 1984). Levels of hyaluronic acid production decrease at the time of condensation and differentiation of limb mesoderm (Toole, 1972, Shambaugh and Elmer,1980; Knudson and Toole, 1985). The mode of interaction of hyaluronate with the cell surface, changes at the onset of mesodermal cell condensation prior to differentiation of cartilage and muscle. Hyaluronate binding sites appear on cells and continues to be present on differentiated chondrocytes but not on myotubes (Knudson and Toole, 1987). Recently Laurent et al (1991) have reported the histochemical distribution of HA in various types of muscle in the rat by using a hyaluronan binding protein (HABP) and the avidin-biotin/peroxidase complex staining procedure. In the skeletal muscle they have observed a heterogeneous distribution of HA. Epimysium and perimysium contained more HA compared with endomysium. However, in muscles with small fiber dimensions (eg. lactoral rectus muscle of the eye, stapedial muscles of the middle ear and tensor tympani), HA staining was very pronounced in endomysium. Specific location of HA in the endomysium may be acting as a cushion separating and

lowering friction between individual muscle fibers. In the cardiac muscle there was more HA in the atrium compared with the ventricle and abundantly present in the perivascular connective tissue. HA was absent in smooth muscle tissue. Boudreau et al (1991) observed the ability of HA to promote migration of ductus arteriosus in culture of smooth muscle cells in culture.

Increased hyaluronic acid levels have been found in several developmental systems involving tissue remodeling, including the formation of chick endocardial cushions (Markwald et al, 1977; Bernanke and Orkin, 1984a, 1984b) cornea (Toole et al ,1984) and mouse neural mesenchyme (Copp and Bernfield, 1988).

Hyaluronic acid binding protein

Hyaluronic acid is enriched in extracellular matrices in which cells migrate or proliferate. There are many evidences which support the role of HA in cellular processes and its influence in cell interactions and differentiation in some cellular systems. Underlying its role in cell behavior is the interactions with hyaluronan-binding proteins (HABPs), both in extracellular matrices and on cell surfaces (Toole, 1990). Recent progress in this field has emphasized the molecular nature and role of HABPs. The two main classes of HABPs: structural HABPs such as HA-binding proteoglycans and link proteins; and cell surface associated HABPs that have properties which suggest that they are HA receptors. These two groups of HABPs have homologous HA binding domains. Recently Toole (1990), has classified them as a single family of proteins and termed them as hyaladherins.

Delpech (1982), first reported the isolation of HABP from human nodes of Ranvier and coined the name hyaluronectin. Girard et al (1982), reported hyaluronectin in human heterografts in the nude mice. After this, several hyaluronic acid binding proteins have been reported, most of which are cell associated ECM glycoproteins with affinity for hyaluronic acid. These proteins have been studied in the brain (Delpech, 1981; D'Souza, 1985), supernatant medium of embryonic heart fibroblasts (Turley, 1982), 3T3 and MSV transformed 3T3 cells (Turley et al, 1987), rat liver (D'Souza, 1986a), brain glial cells (Perides et al, 1989) and rat chondrosarcoma (Crossman and Mason, 1990). There is a separate group of HABPs that apparently serves as cell-surface receptors for HA and mediates the effects of HA on cell behavior. The first of these was originally described as HA-binding sites of high affinity on the surface of SV-3T3 cells (Underhill and Toole, 1979; Underhill and Toole, 1980) and has now been identified in BHK cells as an 85kDa glycoprotein recognized by the K3 monoclonal antibody (Underhill et al, 1987). Another 85kD HABP present at the surface of cultured fibroblasts is derived from serum which has properties distinct from BHK receptor (Yoneda et al, 1990).

An important development is the recent finding of CD44 a hyaluronate receptor same as or very similar to 85kDa HA receptor of BHK cells, present on a wide variety of cells like macrophages, epithelial cells and on some neurons. The glycoprotein has been studied by a number of different laboratories and has been referred as Pgp-1 (Hughes et al, 1981),

Ly-24 (Lynch and Ceredig 1989), ECMR111 (Carter et al, 1989). Subsequent studies have shown that these proteins are closely related or identical to each other (Culty et al, 1990). A HA-binding proteoglycan versican has been identified on fibroblasts. Homology has been observed in the HA binding domain of versican with those of several other HA-binding macromolecules (Zimmerman and Ruoslahti, 1989).

It has been recently reported that the addition of hyaluronate to heart fibroblasts enriched with cellular HA binding protein stimulated the phosphorylation of tyrosine and serine/threonine residues in cellular protein (Turley, 1989a). In another report Turley (1989b), has demonstrated the co-distribution of p^{21} ras protein and HABP in ruffles which might be related to migratory activity and cell surface. In addition, from our laboratory Babu et al (1991), demonstrated the 68kDa HABP associated kinase activity due to autophosphorylation at tyrosine residues and increased phosphorylation in macrophage histiocytomas as compared to normal macrophages. The mentioned reports elucidates the association of tyrosine kinase activity with hyaluronate receptor, suggesting its influence in cellular processes by signal transduction.

Our laboratory reported the presence of naturally occurring HABP in normal rat brain and liver by using HA sepharose affinity chromatography. The molecular weight of the native protein was found to be 68kDa, HABP (Hyaluronectin) was shown to be heat susceptible, protease sensitive and was further characterized as sialic acid containing glycoprotein. The amino acid analysis of

HABP showed that it is rich in glycine and glutamic acid content and is distinct from fibronectin, link protein and gelatin binding protein which are known to bind to hyaluronic acid (D'Souza and Datta, 1986 a,b). Babu et al (1990), demonstrated that 68kDa HABP is a homodimer of 34kDa subunits and that there is a significant increase in levels of 68kDa HABP in diabetic animals. Gupta et al (1991), purified and partially characterized the 68kDa HABP and showed its presence on the surface of confluent, non-permeabilized human fetus lung fibroblasts which was stripped off from the cell surface by urea pretreatment, suggesting that HABP (68kDa) belongs to the same family of cell surface associated hyaluronic acid binding proteins. They have also shown that HABP binds to other extracellular matrix proteins eg. laminin, fibronectin and collagen type IV suggesting the possible role of HABP in the structural organization.

Gupta and Datta (1991), recently described the differential expression of HABP on the cell surface of sub-populations of AK-5 cells. AK-5 a transplantable histiocytic tumor cell line, is a mixture of four different populations and can be grown both as ascites and solid tumors. Cell fractions responsible for developing both ascites and solid tumors contain high amount of hyaluronectin than fractions which are capable of producing only ascites, suggesting its involvement in solid tumor formation.

Even though many reports are available showing the role of HABP in tumorigenesis, phosphorylation, cell interaction, immune system, development etc. no study has been carried out till now

to show the possible involvement of hyaluronectin (HABP) in cell differentiation. We have made a preliminary study to elucidate its role in neonatal muscle cell differentiation in correlation with the antioxidant butylated hydroxy anisole.

Antioxidants

The wide spectrum food additives which are being used have their own impact on environment. Long term exposure of man to chemicals in food might result in deleterious effects as carcinogenicity. The most commonly used food additives are antioxidants.

The widespread use of antioxidants in the food processing industries, especially oil and oil based ones, has great economic advantages. Butylated hydroxy toluene (BHT) and butylated hydroxyanisole (BHA) are the widely used antioxidants in use. Since, the ban on further usage of BHA and BHT by the FAO in 1980, there have been several reports indicating BHA and BHT both having beneficial and detrimental effects. Chronic toxicity have not revealed any adverse effects. Ito et al (1983), have reported the dietary administration of 20,000 ppm of BHA which led to hyperplasia and neoplasia in fore stomach of Fischer strain rats. However, 5000 ppm BHA had a much lower effect. Olsen et al (1983), observed that the first generation offspring in a two generation study involving dietary intake of 250 mg/Kg body weight BHT produced a small but significant yield of hepatocellular adenoma and carcinoma more so in males than in females. Larson and Tarding (1978) reported, intraperitoneal injection of BHT to female mice produced within 3-5 days, a

hypertrophy, hyperplasia and general disorganization of the cellular components of lung. In male mice it was shown that this effect of BHT was associated with a marked stimulation of DNA synthesis in lung (Witschi and Saheb, 1974). Dietary intake of 500 ppm BHT following an initiating treatment with the carcinogen 2-acetyl aminofluorene appeared to exert a promoting effect, not as strong as that of phenobarbitol. Witschi and Lock (1978) observed that BHT enhanced pulmonary carcinogenesis in mice. In a study on BHT inhibition of liver carcinogenesis Williams et al (1973), noted that rats fed 2-acetylamino fluorene, carcinogenesis was inhibited, but at high level of BHT, induction of bladder cancer was seen. Jayalakshmi and Sharma (1986) reported that BHT is more toxic than BHA and the hemolytic activities showed a peak at 60-65% after 12 minutes with BHT and at 50% after 20 minutes with BHA, indicating at the concentrations of 0.75%, BHA and BHT are harmful to the blood mainly affecting erythrocytes which in turn determines their effects on certain cell membranes. In our study we wanted to explore whether these antioxidants affect the cell morphology of myoblasts in vitro and interfere with the differentiation as it has been already reported that it promotes carcinogenesis and effects cell membranes in different tissues.

MATERIALS AND METHODS

Materials

Chemicals

Acrylamide, N-N' methylene bis-acrylamide, bromophenol blue, sodium dodecyl sulphate, β-mercaptoethanol, coomassie brilliant blue R-250 and TEMED were obtained from Sigma chemicals, St. Louis, markers for SDS-PAGE i.e., bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), lactalbumin (14,000) were purchased from Pharmacia Fine Chemicals, Sweden.

TH-3919

AH-sepharose-4B was purchased from Pharmacia Fine Chemicals, Glutaraldehyde, Hyaluronic acid Grade I from human umbilical cord, Agarose, EDTA, BSA were purchased from Sigma Chemicals Co., St. Louis. Sodium azide (LOBA-CHEMIE Indo-Austranal Co., Bombay), Folins' reagent (Bio-rad laboratories), carbozole, sucrose (Analar), butylated hydroxyanisole, cycloheximide, streptomycin, penicillin (Sigma).

Chemicals used in raising polyclonal antisera against HABP

Complete Freunds' adjuvant, and incomplete Freund adjuvant purchased from Difco, Detroit USA.

ELISA and immunoblotting chemicals

Biotinylated goat anti-rabbit IgG, 0.01 M sodium phosphate buffer (pH 7.2), 0.15 M NaCl, 0.05% (w/v) NaN_3 (Bethesda Research

laboratories, BRL)

Streptavidin-alkaline phosphatase conjugate, 0.05 M Tris-HCl (pH 7.2), 0.1 M NaCl, 1 mM $MgCl_2$, 15 mM NaN_3, 40% (v/v) glycerol (BRL), Tween 20 (BRL).

Flat bottom 96 wells microtiter ELISA plates were purchased from Costar, Netherlands.

Goat anti-rabbit IgG conjugated to AP, NBT, BCIP (Promega), Nitrocellulose membrane (Sigma).

Processing of Dialysis tubing

Dialysis tubings obtained from Sigma were boiled for 10 minutes in EDTA (10 mM) solution and then washed with double distilled water.

Maintenance of rats

Wistar male rats were maintained with proper facility and fed with food obtained from Hindustan Lever Ltd.

Chemicals used in heart culture

Ham F10 medium purchased from Gibco

Sodium bicarbonate obtained from Sigma

Fetal calf serum (FCS) and horse serum (HS) were purchased from Seralab, Sussex, England.

25 ml culture flasks and 24 well culture plates were purchased from Costar, Netherlands and trypsinizing flask from Belco, New Jersey, U.S.A.

Methods

Preparation of Hyaluronic acid sepharose-4B affinity column

Hyaluronic acid from umbilical cord (Grade 1, 40 mg) was coupled to glutaraldehyde activated AH-sepharose 4B according to the method described by Cambiasco *et al* (1975).

This is a two step process. The aminated gel is incubated with 25% glutaraldehyde at 4°c for 20 minutes. The unbound glutaraldehyde was removed by washing with 0.01 M PBS, pH 7.2. In the next step glutaraldehyde activated gel was incubated with HA (Grade I from human umbilical cord) in PBS (10 mg/g of gel) at 4°C for 30 minutes. The gel was washed with 0.01M PBS. A further washing was carried out with 0.2 M glycine buffer (pH 8.5). The hyaluronate sepharose was further incubated in 0.2 M glycine buffer, pH 8.5 for 18h at 4°C to block the unreacted aldehyde groups. After washing with PBS the affinity gel was packed into a column of size 0.4 x 15cm.

Hyaluronic acid conjugated to glutaraldehyde activated gel was checked quantitatively. As the quantity of HA with glutaraldehyde activated gel was already known, the amount of hyaluronic acid in the washing was quantified to determine the amount of HA conjugated to glutaraldehyde. We found that around 2mg of HA was bound per ml of swollen gel. Small amount of activated gel in 42% Sucrose and HA in 0.01 M PBS, pH 7.2 dissolved and supernatant was scanned from 200-300 nm, further to confirm the binding of HA to sepharose gel.

Preparation of glycine extract from rat kidney

Purification of HABP was carried out according to the method suggested by Gupta et al (1991). The kidney tissue collected from male wistar rats was weighed and washed with 0.01 M PBS (pH 7.2). The tissue was minced and homogenized in 0.01 M PBS. Homogenate was centrifuged at 12000 rpm for 30 minutes. The pellet was resuspended in double the volume of 0.2 M glycine buffer pH 2.2 and was homogenized. The homogenate was centrifuged at 17,000 rpm for thirty minutes. The supernatant collected was neutralized with 1 N NaOH and the precipitate formed was removed by centrifugation at 12,000 rpm. The supernatant was dialyzed against Phosphate buffered saline, pH 7.2 overnight. This glycine extract was fractionated with 70% saturated ammonium sulfate and the supernatant was dialyzed overnight against PBS.

The dialyzed kidney glycine extract was applied onto the HA Sepharose-4B column and flow rate was maintained (6 drops per minute). After incubation the column was eluted with 0.2 M glycine-HCl pH 2.2. Absorbance was measured at 280 nm. Protein containing fractions were pooled and concentrated in sucrose followed by extensive dialysis against distilled water and 0.01 M PBS, pH 7.2.

Estimation of protein

Protein content was estimated by Lowry's method (Lowry et al 1951), using BSA solution as standard.

Electrophoretic procedure

Sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis was carried out by method of Laemlli (1976). The stacking gel contained 4% acrylamide, 0.106% N-N'methylene bis-acrylamide, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8). The separating gel was made of 12.5% acrylamide, 0.2% N-N'methylene bis-acrylamide, 0.1% SDS and 0.375 M Tris-HCl buffer, pH 8.8. The running buffer was composed of Tris (0.25 M), glycine (0.192 M) and 0.1% SDS, pH 8.3. The sample buffer had the following composition:

Tris-HCl	0.0625 M pH 6.8
SDS	2%
Glycerol	10%
Mercaptoethanol	5% (v/v)
Bromophenol blue	0.001%

Sample protein and low molecular weight standards were applied to the gel and electrophoresed. Protein bands were visualized by silver staining method (Merril et al 1981).

Preparation of polyclonal antibodies to HABP

Polyclonal antibodies to kidney HABP were raised in male New Zealand white rabbits (1.5 Kg). 150 µg of purified HABP was emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly into thigh region of rabbit at multiple sites. Subsequent booster injections of 100 µg HABP emulsified in an equal volume of incomplete Freund's adjuvant were given every fortnight. Animals were bled before each booster dose and the antibody titer was checked by immunodiffusion (Ouchterlony 1962).

After the appearance of best titer value, booster injections were stopped and the serum was collected. The antibody was purified by chromatographing the serum on a Protein A-Sepharose CL-4B column (2ml bed volume, Pharmacia). IgG was eluted with 0.2 M glycine-HCl buffer pH 2.8.

<u>Culture of cardiac myoblast and fibroblast from neonatal rats</u>

Primary culture was carried out according to the method of Harary *et al* (1975). Hearts removed aseptically from 2-3 day old rats were washed and minced in PBSA (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.5 g and KH_2PO_4 0.29 g in 1 litre distilled water) with antibiotics (Streptomycin 50 mg and 30.3 mg of penicillin per 500 ml of PBSA). Minced fragments were transferred into trypsinizing flask (Belco Spinner flask 5000) containing teflon coated magnetic bar. 15 to 20 ml of 0.1% pancreatin in PBSA solution was added and the flask was kept for constant stirring (200 rpm) at 37°C for 10 minutes. The first supernatant collected was discarded as it contains a large number of RBC's, endothelial cells and fragments. The above mentioned dissociation procedure was repeated 4 to 5 times, the cell supernatants collected each time was incubated at 4°C for five minutes followed by the addition of 1 ml of growth medium (Ham F 10) and 10% fetal calf serum to stop the pancreatin activity. The fractions were centrifuged and cells were dispersed by gentle aspiration. The pooled cells were washed thrice further with serum free medium. Finally, the cardiac cells were resuspended in 1 ml of Ham F 10 medium and plated onto petriplates containing 10 ml of Ham F 10 supplemented with 10% FCS, 10% HS, 100 units/ml penicillin and 100 μg/ml streptomycin.

After incubating for 90 minutes at 37^0 C, the contents were transferred to another sterile petri plate and incubated again for 90 minutes to remove the cardiac fibroblasts which gets adhered to the surface. The supernatant enriched with myoblasts was collected and centrifuged, the cells were dispersed by gentle aspiration to make a uniform cell suspension. To a small aliquot of the cell suspension an equal volume of Trypan blue was added (0.4%), and the viability of the cells were ascertained.

Equal number of cells were seeded in 25 ml culture flasks and 24 well plates containing 80% Ham F 10 medium, 10% FCS and 10% HS. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All cultures received fresh medium and serum 24 hours after initial seeding of cells, except for the twenty four well culture plates which received serum free medium. Fibroblasts adhering to the culture flasks were dissociated with 0.1 % pancreatin, washed, counted and seeded as mentioned above. The time when fresh medium was added was considered as zero hour. Cells were frozen and serum free supernatant was collected at regular time intervals (24, 48, 72 h). Cycloheximide was added at a concentration of 25 μg/ml of medium.

1μM to 2μM concentrations of BHA was added along with fresh medium and serum twenty four hour after the initiation of culture. Cells were frozen at 48 and 96 hours after addition of fresh medium.

Cells were harvested with the aid of a rubber policeman, washed twice with PBS 0.01 M, pH 7.2 and dissolved in 0.1 N NaOH. The

suspension was dialyzed against PBS, pH 7.2 and conçentrated with sucrose. Similarly serum free supernatants were concentrated and dialyzed. Protein content in the samples were estimated by Lowry's method.

<u>Enzyme Linked Immunosorbant Assay to quantify HABP</u>

The ELISA for HABP was standardized with some modifications of the method of Engvall (1977). It combines the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays by antibodies coupled to an easily assayed enzyme which also possesses a high turnover number.

Here the double antibody method of ELISA was adopted. Different concentrations (0.02 µg to 1 µg) of HABP in 0.1 M sodium carbonate buffer, pH 9.6 were applied onto the wells of microtiter ELISA plates (96 wells, Costar) for overnight incubation at 4°C. After the binding reaction was over the unbound HABP was removed and the bound HABP was assayed by ELISA using anti-HABP antibody (1: 500) with preformed complex (50 µl of biotinylated secondary antibody, goat AR-IgG, 1:200 dilution and 50 µl of Streptavidin-alkaline phosphatase conjugate, 1:200 dilution) in 10 ml of 1% BSA in TBST .

After every step the plate was washed extensively with TBS-Tween 20. The colour was developed by addition of 100 µl per well of substrate solution (40 mg of pNPP in 10 ml of substrate buffer with 100 mM Tris, 100 mM NaCl and 50 mM $MgCl_2.6H_2O$, pH 9.5) and the reaction was stopped by adding 25 µl of 1N NaOH. The plate was read at 405 nm in a micro ELISA reader. A standard graph was

plotted (Figure 1). Same procedure was carried for all extracts and supernatants (Normal, with cycloheximide, with BHA) and HABP was quantitated from the standard curve.

Immunoblotting

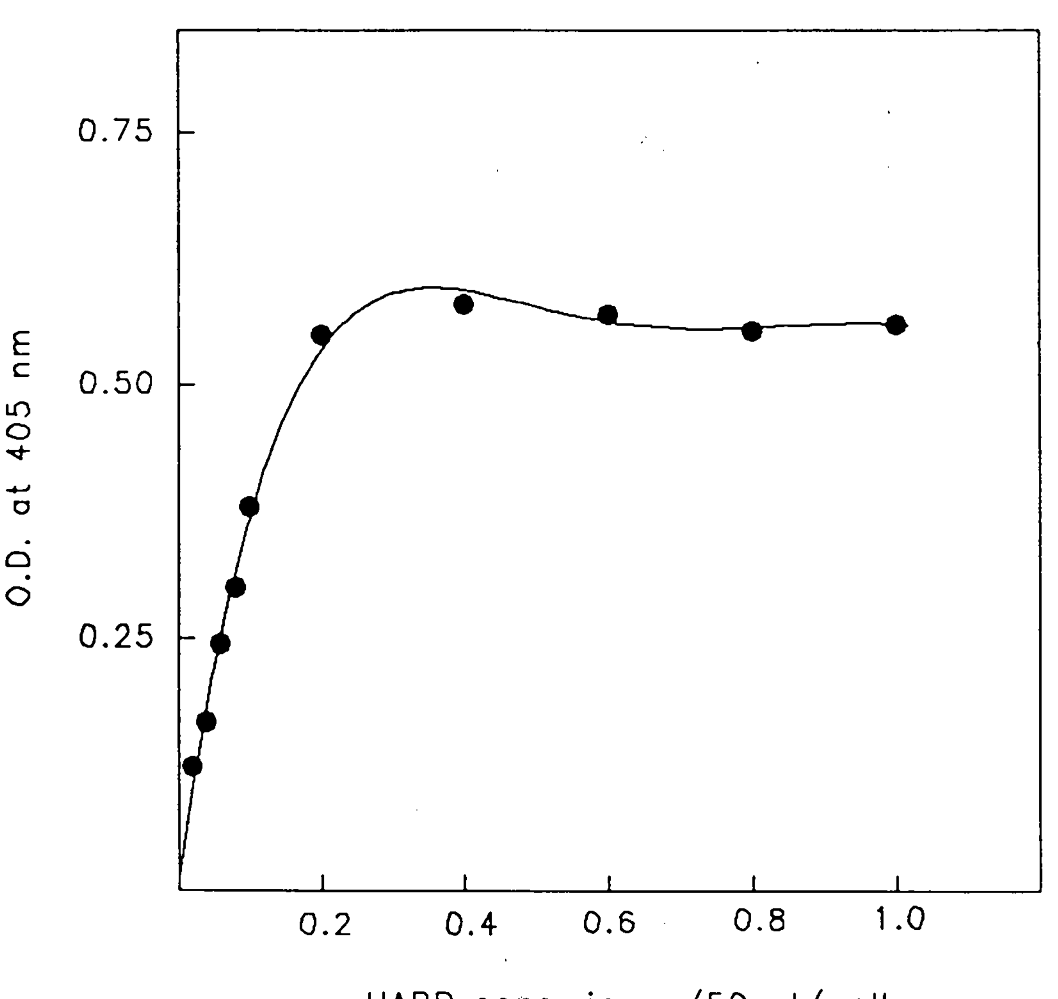
Purified HABP, cell extracts and supernatants were electrophoresed on 12.5% discontinuous SDS-PAGE and then electrophoretically transferred (Towbin et al , 1974) on to nitrocellulose sheets. After washing briefly in PBST, the sheet was incubated in 2% BSA for three hours at 37°C to reduce the background labeling. After washing with PBS containing 0.05% Tween 20 for one hour at room temperature it was incubated with polyclonal antibodies raised against HABP (1:500 dilution) for one hour followed by the washings (four times) with PBST. The nitrocellulose paper was incubated with goat anti-rabbit IgG conjugated to AP (1:7500 dilution) for one hour at 37°C. After thorough washing in PBST to remove the excess antibody the sheets were transferred to color developing solution [66 µl NBT and 33 µl BCIP in 10 ml of AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM Nacl and 5 mM $MgCl_2$)]. The sheets placed in the above solution were gently rocked and removed when the purple color developed in the background.

FIGURE 1

Assay system for HABP by ELISA

Varying concentrations of protein (0.02 μg - 1 μg) in the final volume of 50 μl of 0.1 M sodium carbonate buffer, pH 9.6, were coated onto microtiter plates. After washing the wells were incubated with anti-HABP (1:500) antibodies, then with preformed complex of biotinylated goat anti-rabbit IgG (1:200) and streptavidin-alkaline phosphatase conjugate (1:200) as mentioned under materials and methods. Colour was developed by incubating the wells with substrate and read at 405 nm in an ELISA reader. Values represent the mean ± S.D. of 6 replicates.

Figure 1



RESULTS

Homogenous purification of HA binding protein

HA binding protein has been purified according to the method described earlier (Gupta *et al*, 1991) using HA-sepharose affinity column. The binding protein revealed a single band of molecular weight 34kDa in reduced SDS-PAGE analysis (Figure 2). We raised polyclonal antibodies against this purified protein in rabbits and checked its monospecificity by western blot analysis of cell extracts, which yielded single bands at 34kDa. Thus we could use the antibodies for our further experiments.

Evidence for the presence of HABP in neonatal rat cardiac myoblast and fibroblast cultures

Using the polyclonal antibodies directed against HABP we tried to detect the presence of this protein in rat cardiac myoblasts and fibroblasts by immunoblot analysis. As shown in Figure 3, we could detect only a single band at 34 kDa which is identical to that of purified HABP, in cell extracts of myoblasts and fibroblasts. Preliminary observation which has been made is that the signal is stronger in one day old myoblast culture when compared to three day old cultures. The band corresponding to the HA binding protein is more prominent in the fibroblast culture as compared to myoblasts. Equal amount of total protein (12 µg) was taken of the cell extracts.

Secretory nature of HABP in relation to myoblast differentiation

The serum free supernatant medium from different day old cultures of cardiac myoblasts and fibroblasts were subjected to western

FIGURE 2

Subunit molecular weight of HABP by SDS-PAGE

Lane 1: Marker proteins [phosphorylase b (94kDa), BSA (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20kDa) and lactalbumin (14.4kDa).

Lane 2: 4 μg pure HABP in the absence of β-mercaptoethanol.

Lane 3: 4 μg pure HABP in the presence of β-mercaptoethanol.

Electrophoresis was carried out at the constant volatage of 80 v in 12.5% gel in the presence of SDS and protein bands were visualized by silver staining.

Fig. 2

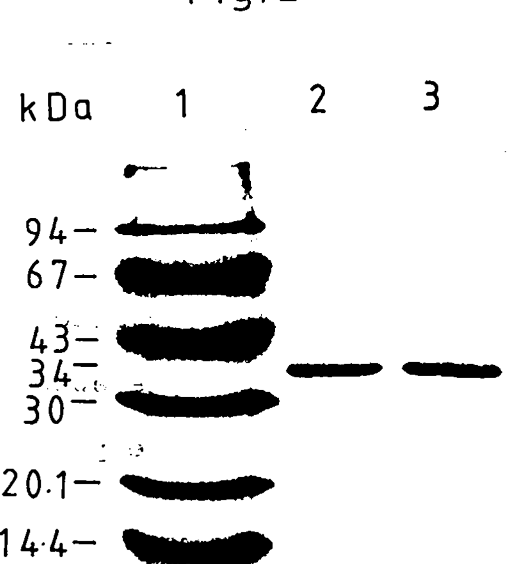


FIGURE 3

Presence of HABP in neonatal rat cardiac myoblasts and fibroblasts.

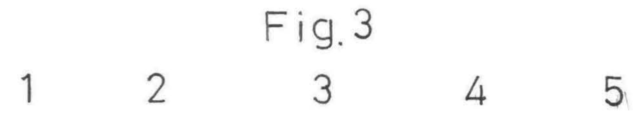
12 μg of protein from cell extracts of cardiac myoblasts grown for 24 h after the addition of fresh medium (Lane 1) and 72 h (Lane 2); 12 μg of protein from cell extracts of cardiac fibroblasts grown for 24 h (Lane 3) and 72 h (Lane 4); 6 μg of pure HABP from rat kidney (Lane 5) subjected to 12.5% SDS-PAGE gel electrophoresis. Proteins in the gel were electrophorectically transferred onto NC sheet. Blotted proteins were incubated with anti-HABP antibodies and then visualized as mentioned in materials and methods.

FIGURE 4

Evidence for HABP as secretory protein

85 μg of proteins from serum free supernatant culture medium of neonatal cardiac myoblasts collected after 24 h after the addition of fresh medium (Lane 2), 48 h (Lane 3) and 72 h (Lane 4); serum free supernatant from 24 h fibroblasts cultures (Lane 1) and 5 μg of pure HABP from rat kidney (Lane 5) were subjected to 12.5% SDS-PAGE. Proteins in the gel were electrophoretically transferred onto NC sheets, blotted protein were incubated with anti-HABP antibodies and then visualized as mentioned in materials and methods.

Fig. 3



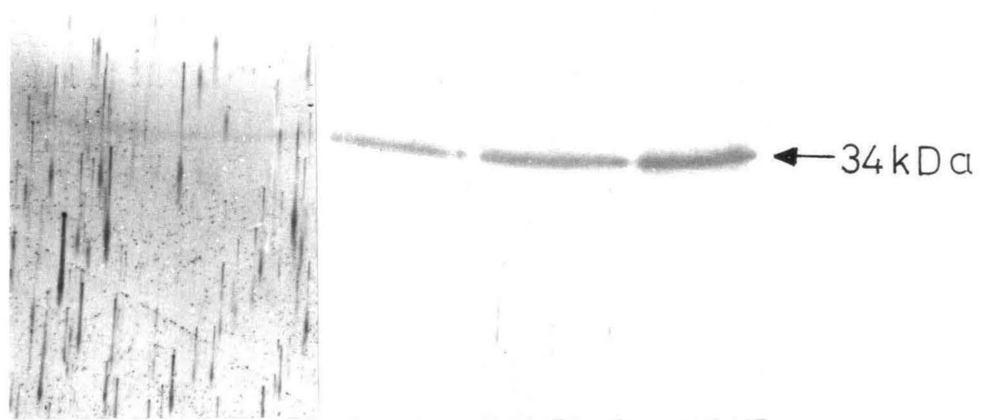


Fig. 4

5 4 3 2 1

34 kDa

blot analysis by taking equal quantity (85 μg) of total protein. The significant difference in the amount of HABP can be visualized by the difference in the intensity of the bands. Figure 4 shows, an increase in the level of secretory HABP with increasing age of cardiac myoblasts in culture. We can also visualize, the secretory HABP level is much higher in the supernatant of the fibroblasts than myoblasts by looking at the intensity of the bands.

<u>Changes in the protein profile in the cell extracts and serum free supernatant medium during myoblast differentiation</u>

The total protein content in differentiating myoblast cultures and serum free supernatant medium, in the presence and absence of cycloheximide was determined from standard curve using BSA (Figure 5 and 6).

Under normal conditions total protein content is showing a rapid increase from second to third day in cell extracts (Figure 5). In contrast, in the presence of cycloheximide there is a gradual decline in the total protein concentration which is due to inhibition of protein synthesis.

In the serum free supernatant medium, total protein concentration is increasing linearly with myoblast differentiation (Figure 6), whereas in the presence of cycloheximide there is a decrease in the level from first to second day followed by an increase from second to third day. This could have been due to cell death which leads to the release of cellular contents in the medium.

FIGURE 5

Total protein profile of cell extracts of myoblasts in culture

Protein content of cell extract of myoblasts during differentiation in the culture was estimated as mentioned in materials and methods. Total protein content is represented in µg/ml. ○—○ represents the culture under normal condition with no additions, ●—● represents the cultures to which cycloheximide (25 µg/ml) was added. Each point represents the mean ± S.D. of triplicates.

FIGURE 6

Total protein profile of serum free supernatant medium of myoblast in culture

Protein content of serum free supernatant medium of myoblasts during differentiation in the culture was estimated as mentioned in materials and methods. Total protein content is represented in mg/ml. ○—○ represents the culture under normal condition with no additions. ●—● represents the cultures to which cycloheximide (25 µg/ml) was added. Each point represents the mean ± S.D. of triplicates.

Figure 5

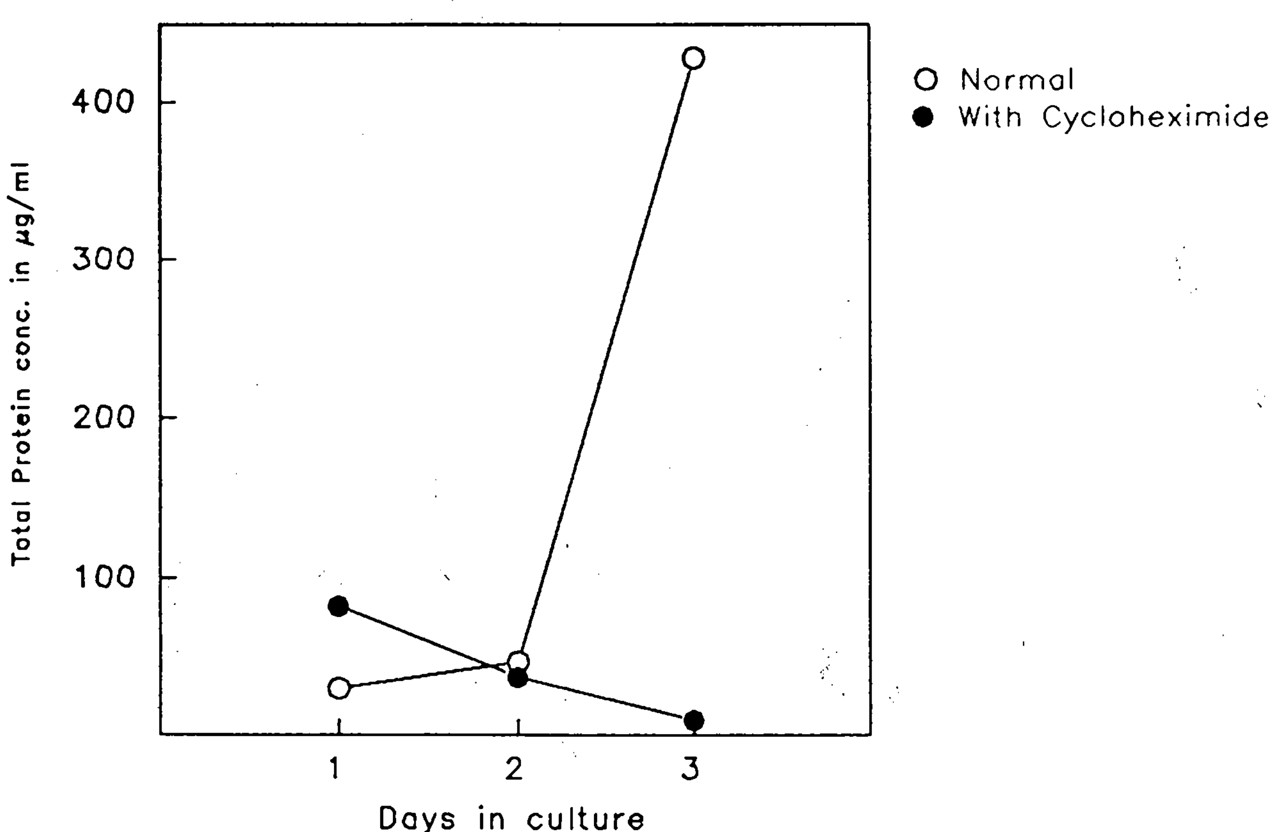
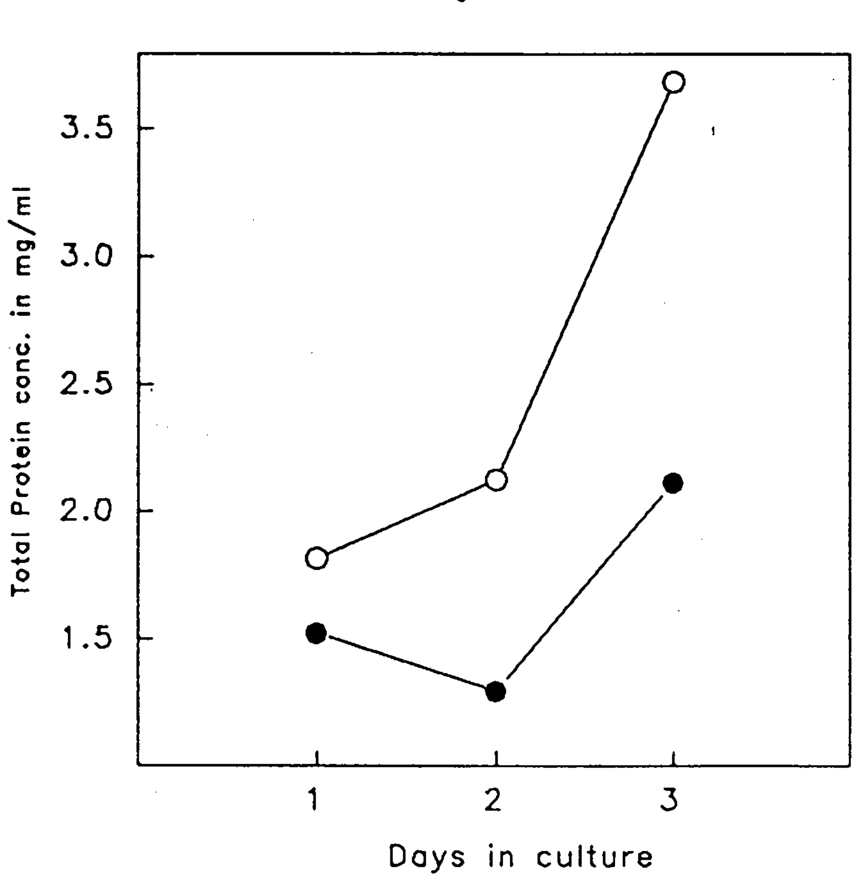


Figure 6



Changes in the level of hyaluronectin (HABP) during myogenesis

To examine the changes in the level of HABP during myoblast differentiation, the ELISA technique was employed. The concentration of HABP in μg/mg of total protein was calculated from the standard curve of HABP (Fig 1). As shown in figure 7a, level of HABP in myoblast cell extracts declined with days in culture. In contrast the level of HABP in serum free supernatant medium was observed to increase with time (Fig. 7b), whereas in the presence of cycloheximide (25 μg/ml) HABP showed a declining pattern both in cell extracts and supernatant medium.

Comparison of HABP level in cell extract and supernatant medium during myoblast differentiation

As shown in Figure 8, the concentration of HABP in cell extracts was almost 100 times higher when compared to serum free medium. This suggests that majority of HABP molecules secreted out might be binding to cell surface.

The Changes in the cell morphology of myoblast in the presence of PHA

To study the effect of BHA in neonatal cardiac myoblasts, the cells were grown in medium with serum (control cultures) and in medium containing 2 μM BHA after growing the cells in normal medium with serum for 24 h (experimental cultures). As presented in Figure 9a, the myoblasts (control cultures) do not fuse but form a monolayer culture. The contraction of the cardiac myoblasts were observed under the microscope which increased with the progression of myogenesis. As shown in Figure 10a-10c,

FIGURE 7a

Changes in the HABP level in cells during progression of myogenesis

HABP contents in the cell extracts of myoblasts were assayed by ELISA technique as mentioned in materials and methods, represented in µg/mg of total cell proteins. ○——○ represents cultures under normal conditions with no additions, ●——● represents cultures to which cycloheximide (25 µg/ml) was added. Each point represents the mean ± S.D. of 6 replicates.

FIGURE 7b

Changes in the HABP level in serum free supernatant medium during progression of myoblast differentiation in culture

HABP contents in serum free supernatant medium of myoblasts were assayed by ELISA technique and represented in µg/mg of total secreted proteins. ○——○ represents culture under normal condition with no additions. ●——● represents the cultures to which cycloheximide (25 µg/ml) was added. Each point represents the mean ± S.D. of 6 replicates.

Figure 7a

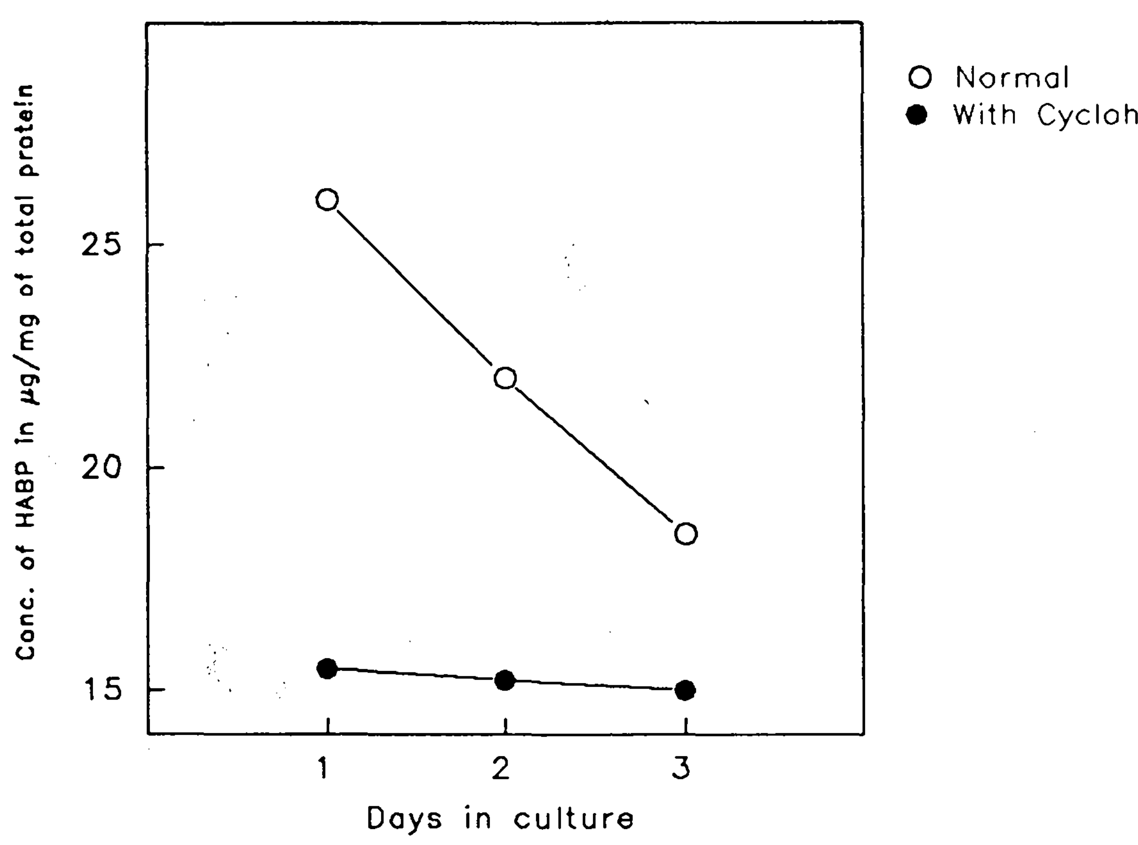


Figure 7b

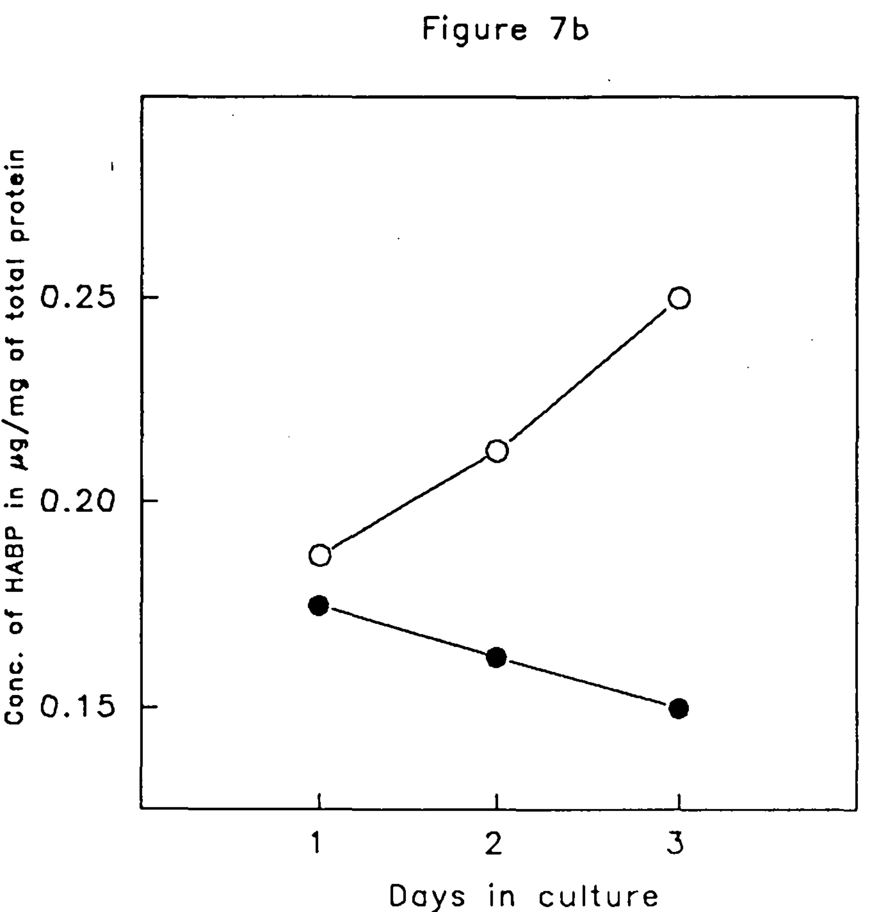


FIGURE 8

This histogram illustrates the comparison between HABP levels in cell extracts (▧) and serum free supernatant medium (▥) of myoblasts in culture. Values represent the mean ± S.D. of six replicates.

Figure 8

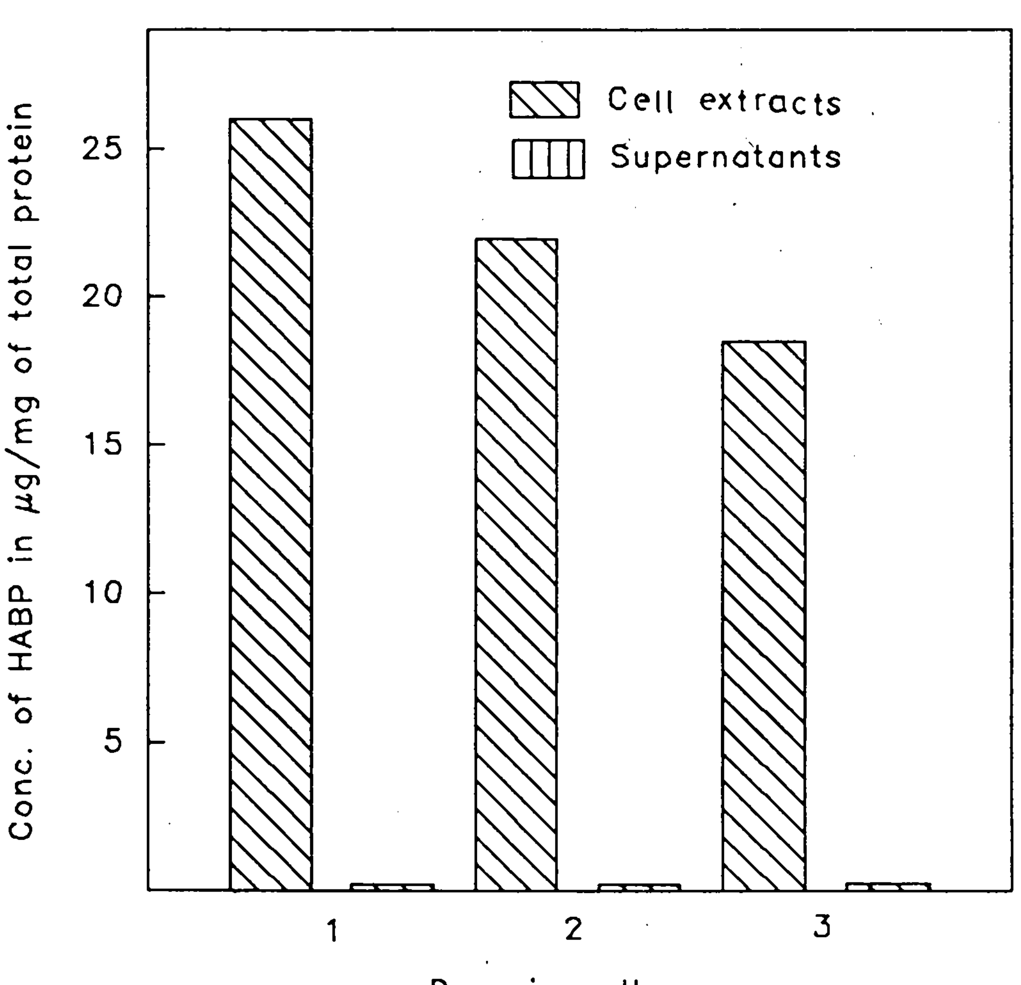
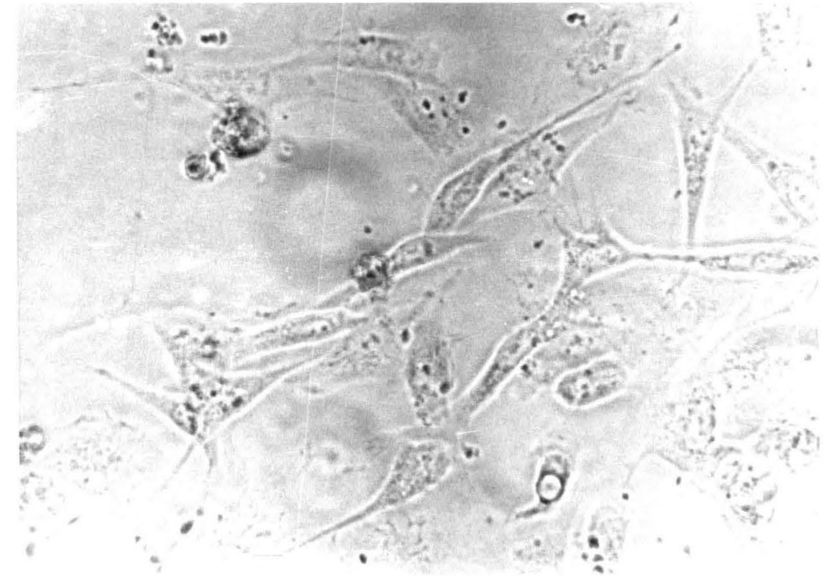


FIGURE 9

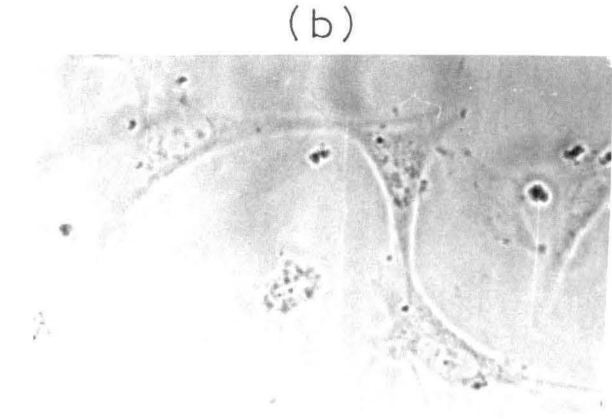
Phase contrast microscopic photograph illustrating the myoblast enriched culture with 10-20% of fibroblasts (a), and pure fibroblasts culture (b).

Fig. 9

(a)



(b)



myoblasts gradually became elongated with days in culture. In the presence of 2 μM BHA though there was no significant change in cell morphology (Figure 10d-10f), the cells were more elongated during differentiation. We have also observed an increase in the number of beating cells in the presence of BHA.

The changes in HABP level during myoblast differentiation in relation to presence of antioxidant BHA

As we have seen the differential expression of HA binding protein during heart cell biogenesis, we were interested to examine the effect of BHA on HABP level during the myogenesis. As shown in Figure 11, we have estimated the level of HA binding protein in BHA exposed cells on different days of culture (2 and 4 days). It is interesting to note that in 2 μM concentration of BHA, the level of HA binding protein in myoblasts did not decrease as occured in four days old control cultures.

FIGURE 10

Phase contrast microscopic photographs illustrating the cell morphology of myoblasts on different days culture. (a) - 1 day; control, (b) - 3 days; control, (c) - 4 days; control, (d) - 1 day; with 2 μM BHA, (e) - 3 days; with 2 μM BHA and (f) - 4 days; with 2 μM BHA.

Fig. 10

(a)

(d)

(b)

(e)

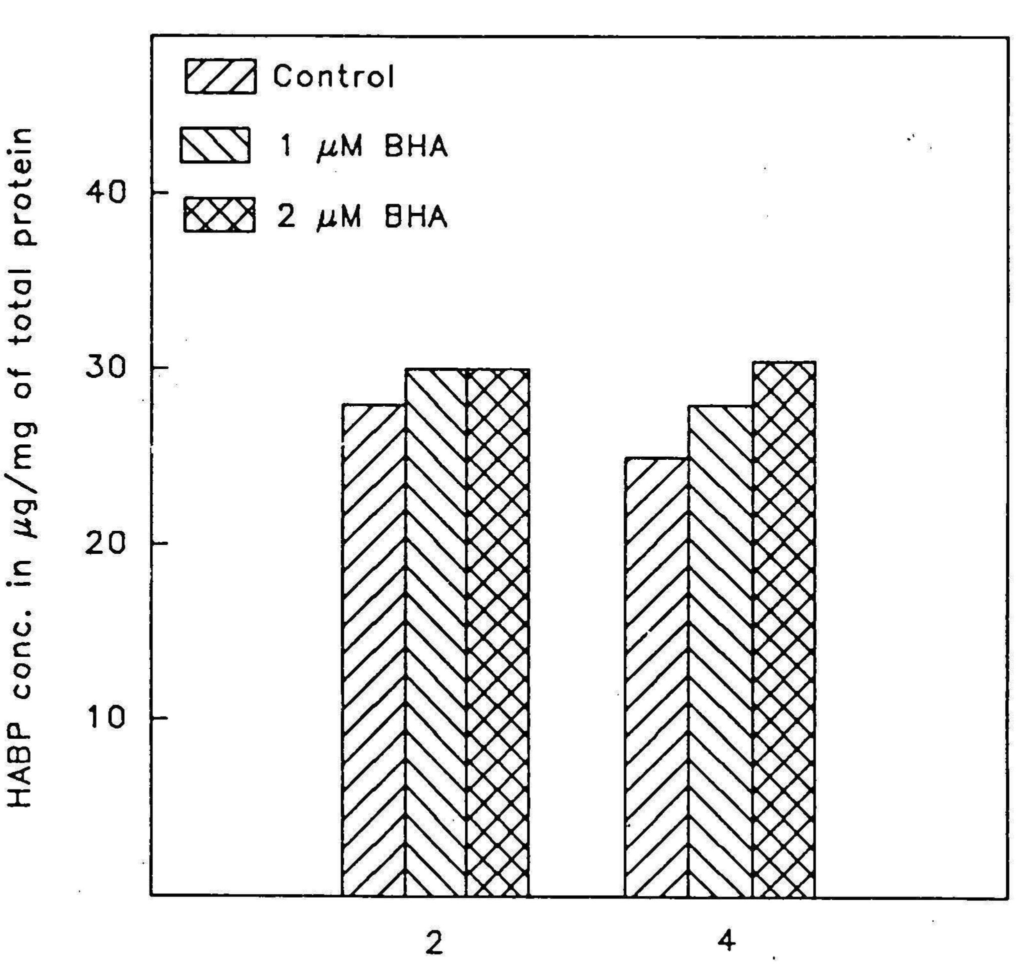
(c)

(f)

FIGURE 11

This histogram illustrates the effect of different concentrations of antioxidant BHA on the changes in the level of HABP during differentiation of myoblasts in culture. [hatched box] represents the control cultures with no added antioxidant, [hatched box] represents to which 1 μM of BHA was added and [cross-hatched box] represents cultures to which 2 μM concentration of BHA was added. Values represent the mean ± S.D. of 4 replicates.

Figure 11



DISCUSSION

Our present report documents the occurrence of a 68kDa (a homodimer of 34kDa subunits) HABP in the neonatal rat cardiac myoblasts and fibroblasts. Evidences presented in this study suggests that the 68kDa rat cardiac myogenic and fibrogenic cells derived HABP is identical or very similar to rat tissue derived HABP with respect to molecular weight and immunological characteristics. Further, we show the secretion of HABP in the culture medium of neonatal rat cardiac myoblasts and fibroblasts. The secretion of HABP in the culture medium was totally blocked when the cells were cultured in the presence of cycloheximide (25 µg/ml). This data confirms that HABP secreted in the medium is synthesized <u>de novo</u> in myoblasts and fibroblasts. Our interest was to elucidate the physiological role of HABP during the progression of myogenesis. The results which we have obtained are showing a gradual decline in the myoblast HABP level during differentiation with concomitant increase in the secretion of HABP in the medium. This suggests its possible involvement in myogenesis. Our results may be further extended to show the HABP interaction with its ligand i.e., HA which may be associated in myoblast differentiation. This observation is supported by the report of Toole <u>et al</u> (1984), that hyaluronan can expand tissue spaces and alter physical restraints to movement. Another important observation was made by Knudson and Toole (1987) on the mode of interaction of hyaluronate with cell surface which changed at the onset of differentiation of chick embryo limb mesoderm. The hyaluronate binding sites appeared and continued to

be present on differentiated chondrocytes but decreased in myotubes. Recently, Laurent et al (1991), have elucidated that HA occurs at sites subjected to pronounced mechanical stress. He showed the presence of HA around cardiac muscle fibers and on endomysium of skeletal muscle where it acts as a cushion separating and lowering the friction between the individual fibres.

Unlike the skeletal myoblasts, cardiac myoblasts do not fuse, they arrange themselves to form fibres, which means the muscle cells need to be tightly bound to each other in order to function and contract as a unit and the presence of HA might inhibit this process due to its hydrophobic nature. The function of HA binding protein is always in correlation with the hyaluronic acid. The reduction in the level of HA and its binding sites during myogenesis can be attributed to the decrease in the levels of HABP during cardiac myoblast differentiation. Similar paradigm has been observed by Chung and Kang (1990) in fibronectin. They suggested the decrease in fibronectin level during myogenesis was in correlation with the alteration of its receptor and with fusion of myoblasts.

In the immunoblot analysis, we have observed that the level of 68kDa HABP, a novel ECM protein, is much higher in fibroblasts as compared to myoblasts. Chiquet et al (1981), has also reported that compared to fibroblasts, myoblasts and myotubes synthesize little fibronectin of their own and accumulate little on their surfaces. As muscular tissues are composed of a heterogeneous

cell population consisting mainly of myoblasts and fibroblasts, our findings suggest that certain extracellular components specifically HABP and fibronectin might be contributed more by the fibroblasts compared to myoblasts to ECM of muscular tissues. It is important to mention here that from our laboratory, the interaction of HABP with fibronectin has been reported (Gupta et al, 1991) and we speculate its involvement in structural organization of the tissues.

Another important observation made was that the amount of HABP secreted in the culture medium of myoblasts was significantly lower than the level of cellular HABP present intracellularly and/or associated with the cell surfaces of myoblasts. This might be due to the rapid degradation of secreted HABP molecules which are not bound to the cell surface.

Although the level of HA binding protein during myogenesis is reduced, the exact cause of its mechanism is not known. There are possibilities that the reduction of HA in myoblast differentiation might be regulating the rate of synthesis of HABP. It is also possible that during the process of differentiation, the secretory rates are altered. In order to confirm our speculation, we would like to study the cell surface localization of HABP during myoblast differentiation. We are also interested in examining the rate of synthesis and process of glycosylation using ^{35}S-methionine in the medium.

In the presence of BHA (2μM), myoblasts did not exhibit the decreasing pattern of HABP as observed in the control. It shows

that this antioxidant might be interfering with the cardiac biogenesis. The addition of BHA to the culture medium seems to alter the cell shape and structure. This is a preliminary study, it is essential to pursue in this field and unravel the toxic effects of BHA which is a controversial environmental issue.

In summary, our findings suggest that 68 kDa HABP might be playing a physiologically significant role in myogenesis and its function is disturbed by the addition of external environmental agents.

SUMMARY

Hyaluronic acid binding protein (hyaluronectin) has been purified to homogeneity from normal adult rat kidney by hyaluronate sepharose affinity chromatography. SDS-PAGE analysis of HABP under reducing as well as non-reducing conditions revealed a single protein band of molecular weight of 34kDa. Polyclonal antisera was raised against it and the monospecificity of the antibodies towards HABP was confirmed by Western Blot analysis of cell extracts. Immunoblot analysis has elucidated the occurrence of this glycoprotein in neonatal cardiac myoblasts and fibroblasts. Quantitative analysis by ELISA technique has revealed the declining pattern of HABP in myoblasts during differentiation and vice versa in the serum free supernatant medium of myoblast cultures. We have also shown the increased level of HABP in cardiac fibroblasts as compared to myoblasts and significantly low level of this glycoprotein in serum free supernatant medium compared to cell extracts of myoblasts. This observed pattern of HABP concentration during myogenesis was disturbed by the addition of BHA, an impoitant antioxidant under controversy.

Our results suggest the possible involvement of hyaluronectin in cardiac myogenesis and interference by external environmental agents. Future experiments will enable us to unravel the exact mechanism involved.

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