Immunohistochemical Demonstration of S-100 Protein in Fibroblast-Like Cells of the Guinea-Pig Heart

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Received December 2, 1985

Summary. The immunocytochemical localization of S-100 protein was examined in the hearts of adult guinea-pigs. In addition to Schwann cells, adipose cells and chondrocytes, fibroblast-like cells densely distributed in the cardiac skeleton, all of the four valves and tendinous chordae were immunoreactive for S-100 protein. The S-100-positive fibroblast-like cells were conspicuously rounded in shape and extended their thin, thread-like cell processes in opposite directions; the cytoplasm was restricted to perinuclear region. In these morphological features, the cells were distinctly different from ordinary fibroblasts which were immunonegative for S-100 protein. Electron microscopic observation revealed that the S-100-like immunoreactivity was distributed throughout the cytoplasmic matrix in the cell body and processes, but absent from the nuclei and cell organelles. The significance of the S-100 immunopositive fibroblast-like cells was discussed in relation to cartilaginous tissues, which have the potential to form in the region of the cardiac skeleton and valves under both normal and abnormal conditions.

S-100 protein was first isolated by Moore (1965), from the brains of various animals. This protein is characterized by its solubility in saturated ammonium sulphate at pH 7 and predominant existence in nervous systems (cf. Bock, 1978). It belongs to the family of Ca2+ binding proteins such as calmodulin and troponin C (Isobe and Okuyama, 1978). Immunohistochemical studies have revealed that the protein is primarily contained in glial elements in the central and peripheral nervous systems (Cocchia, 1981; Stefansson et al., 1982a). Some glia-like cells in paraneuronal tissues have also shown the immunoreactivity for S-100 protein. This group includes folliculo-stellate cells in the anterior pituitary and sustentacular cells in the carotid body and adrenal medulla (Nakajima et al., 1980; Cocchia and Miani, 1980; Cocchia and Michetti, 1981; Kondo et al., 1982).

The immunoreactivity for S-100 protein, however, was also found to occur in a variety of cells which are not related to glial cells. Nakajima et al. (1982) reported that, in human, the immunoreactivity was localized in Langerhans cells in the skin, interdigitating reticulum cells in the lymph nodes and thymus, and histiocytosis X cells. In the rat lymph node, however, the S-100-immunoreactivity was restricted to reticular cells within the germinal center (Iwanaga et al., 1982). Furthermore, chondrocytes and fat cells in various mammals were found to be intensely immunoreactive for S-100 protein (Stefansson et al., 1982b; Suzuki et al., 1982; Michetti et al., 1983). Recently, S-100-like immunoreactivity was recognized in myoepithelial cells in exocrine glands.
(HARA et al., 1983; MOLIN et al., 1984). It is noteworthy that the subcellular localization of S-100-like immunoreactivity is invariable among cell types: the immunoreaction products for S-100 protein are evenly distributed in the cytoplasmic matrix, leaving spaces for cell organelles.

According to the amino acid analysis of S-100 protein, this protein comprises three forms, S-100αo, S-100α and S-100β, which are dimers with the subunit composition of αα, αβ and ββ, respectively (cf. ISOBE et al., 1984). In the bovine brain, S-100α and S-100β are dominant, and S-100αo is a minor component (ISOBE et al., 1983). TAKAHASHI et al. (1984) immunocytochemically revealed that β subunit immunoreactivity was present in all S-100-positive cells reported previously, while immunoreactivity for the α subunit was restricted to some of them. Other immunological and immunocytochemical studies have reported that neurons, urinary tubules and cardiac and skeletal muscles were immunoreactive for the α subunit only; namely, they contained S-100αo (ISOBE et al., 1984; KATO and KIMURA, 1985).

Now it is clear that S-100 protein is not specific for glial elements, but that a variety of cells of different origin and function contain this protein. Nevertheless, its peculiar distribution in certain distinct categories of cells is obvious.

In the present study, we report on the localization of S-100-like immunoreactivity to a special population of fibroblast-like cells in the heart of the guinea-pig.

MATERIALS AND METHODS

The antiserum used was raised in rabbits using an S-100 protein preparation isolated from bovine brains as the antigen (MASUDA et al., 1983). In previous studies of our research group, this antiserum was shown to not stain any neuronal elements or muscles, suggesting that the antiserum was predominantly specific to the β subunit (USHIKI et al., 1984; IWANAGA et al., 1985).

Eight adult male guinea-pigs, weighing about 300 g, were used in this study. After the animals were killed by intraperitoneally injecting an overdose of sodium pentobarbiturate, their hearts were removed. Bouin’s fixative of 20 ml was injected into both the right and left ventricular cavities through the cardiac apex and then immersed in the same fixative for additional 6 hrs. The dissected tissue blocks were dehydrated through a graded series of ethanol and embedded in paraffin. Paraffin sections were cut at 4-5 μm thickness. Dewaxed paraffin sections were submitted to the peroxidase-antiperoxidase (PAP) method (STERNBERGER, 1974) using an anti-S-100 serum diluted at 1:1,000. The sections were incubated with the diluted antiserum for 12 hrs or more at room temperature. They were rinsed in phosphate-buffered saline and incubated with swine anti-rabbit IgG (Dakopatts, Denmark) diluted 1:60 for 40 min. The sections were then incubated for 40 min with PAP complex (Dakopatts, Denmark) diluted at 1:120. After rinsing, an enzyme reaction was developed with diaminobenzidine, sometimes being counterstained with hematoxylin.

For electron microscopic immunocytochemistry, the hearts were fixed with an intracardiac injection of 4% paraformaldehyde in 0.1 M phosphate buffer (about 20 ml), pH 7.4 and immersed in the same fixative for 6 hrs. The tissue blocks were dehydrated through a graded series of ethanol and embedded in paraffin. Paraffin sections were cut at 4-5 μm thickness. Dewaxed paraffin sections were submitted to the peroxidase-antiperoxidase (PAP) method (STERNBERGER, 1974) using an anti-S-100 serum diluted at 1:1,000. The sections were incubated with the diluted antiserum for 12 hrs or more at room temperature. They were rinsed in phosphate-buffered saline and incubated with swine anti-rabbit IgG (Dakopatts, Denmark) diluted 1:60 for 40 min. The sections were then incubated for 40 min with PAP complex (Dakopatts, Denmark) diluted at 1:120. After rinsing, an enzyme reaction was developed with diaminobenzidine, sometimes being counterstained with hematoxylin.

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Fig. 1. The atrioventricular junction of the guinea-pig heart. PAP staining by use of S-100 protein antiserum. Numerous S-100-immunopositive cells (arrows) zonally spread in the connective tissue, continuing into the atrioventricular valves. MV mitral valve, IAS interatrial septum, IVS interventricular septum. Counterstain with hematoxylin. ×55

Fig. 2. High magnification of a part of Figure 1 (the area enclosed within the square). Most of the S-100-immunopositive cells are oval or spherical in shape. Cardiac muscle cells (arrows) dispersed in the loose connective tissue are almost negative in reaction. ×550
tetroxide in 0.1 M phosphate buffer for 5 min and were stained in 2% uranyl acetate for 10 min. Following this, they were dehydrated and embedded in Araldite. Ultra-thin sections were examined with an electron microscope (Hitachi, HU 125 DS).

The specificity of the immunostaining was checked by a preincubation of the antiserum with the antigen (S-100 protein 50 μg/ml diluted antiserum). No immunoreactivity for S-100 protein was recognized in the sections stained with the antigen-absorbed antiserum.

RESULTS

The immunoreactivity for S-100 protein was recognized in nervous elements distributed throughout the heart. It was localized in Schwann cells associated with nerve fibers and in satellite cells surrounding neuronal somata. The immunoreactivity was also found in adipose cells and in chondrocytes occasionally present at the base of the aortic wall. Cardiac muscles appeared to be slightly positive in reaction. When their stainability was compared with that in the absorption test, it was apparent that a weak immunoreactivity for S-100 protein was present in the cardiac muscles (Fig. 1, 3).

In addition, it was noticed that S-100 positive cells densely occurred both in the cardiac skeleton and in all of the four cardiac valves (Fig. 1, 3). Although these S-100 positive cells might be classified into fibroblasts, they were different in some morphological characteristics from typical fibroblasts which were located in the atrial and ventricular myocardium as well as pericardial and endocardial connective tissue, and which were negative in immunoreaction. The nuclei of the S-100 positive cells were conspicuously rounded in shape as compared with the ordinary fibroblasts (Fig. 4). The cell-bodies were oval or thick spindle-shaped: when crossly or obliquely sectioned, they appeared to be globular in shape (Fig. 2, 6). In longitudinal profile, the cytoplasmic processes which extended in opposite directions suddenly tapered into thread-like processes (Fig. 4). The immunoreactivity for S-100 protein was found throughout the cytoplasm including the cell processes, while it was absent from the nucleus.

The cardiac skeleton is composed, for the most part, of dense connective tissue, its main components being the membranous septum, the fibrous trigones and the annular portion of the valves. The S-100 positive fibroblast-like cells were widely distributed with a zonal arrangement throughout the cardiac skeleton (Fig. 1). At the base of the atrioventricular valves, they continued to a population of S-100 positive cells in the zona spongiosa of the valves. In the annular portion of the semilunar valves, S-100 positive cells were arranged most densely in a space between the aortic or pulmonary arterial wall and ventricular myocardium (Fig. 3, 4).

The cardiac valves are covered with a single layer of endothelial cells; the stroma of the valves is occupied by a comparatively loose connective tissue called the zona spongiosa, which is composed of scattered fibroblasts and collagen fibrils. Most of the fibroblasts existing in the zona spongiosa of all the four valves were immunoreactive for S-100 protein (Fig. 5, 6). The valvular S-100 positive cells possessed elongated nuclei and wide-spread cytoplasmic processes, presenting a similarity to typical fibroblasts (Fig. 5). In the middle and distal thirds of the valves, the stromal S-100 positive cells were densely distributed and showed an especially strong immunoreactivity (Fig. 6), while in the proximal third of the valves they decreased in number and in intensity of the immunoreaction. In the proximal two thirds of the valves, the zona fibrosa composed of denser connective tissue occurred on the ventricular side of the atrio-
Fig. 3. A dense accumulation of S-100-immunopositive cells is seen in the annular portion of the pulmonary valve (PV). Cardiac muscle cells of the right ventricle (RV) are weakly positive in reaction. Immunoreactive elements (arrows) at the base of the pulmonary artery (PA) are glial cells associated with neurons. ×95

Fig. 4. High magnification of a part of Figure 3 (the area enclosed within the square). Immunoreaction for S-100 protein is present in the perinuclear cytoplasm and its slender processes, but absent from the round nucleus. ×850
ventricular valves and in the arterial side in the semilunar valves (Fig. 5). Although a small number of typical fibroblasts was scattered among the dense collagen layer in zona fibrosa, they were negative in reaction.

The stroma of the chordae tendineae of atrioventricular valves also contained numerous S-100 positive fibroblast-like cells. The S-100 positive cells were found throughout the chordal stroma from the valvular to the papillary end. The immunoreactivity in the chordae tendineae, however, was less intense than that in the valves.

In the connective tissues of the cardiac skeleton and valves, occasional cells named the Anitschkow cell or caterpillar cell were found intermingling with fibroblasts. They were characterized by a peculiar nucleus, in which chromatin accumulated into a rod-shape in the center. Several Anitschkow cells showed a positive reaction to the anti-S-100 serum in their cytoplasm (Fig. 7), but the major part of Anitschkow cells was free from the S-100-like immunoreactivity.

Electron-microscopic observation revealed the ultrastructure of S-100-immunoreactive cells marked by an electron-dense reaction product (Fig. 8). The S-100 positive cells were dispersed in the stroma of the valves and in the cardiac skeleton, intermingling with collagen fibers and elastic lamellae. They had a round or oval nucleus with slightly indented surface and poorly developed perinuclear cytoplasm. The immunoreactive products for S-100 protein were distributed throughout the cytoplasmic matrix, while little or no reaction products were present in the karyoplasm. Thread-like cytoplasmic processes, characteristic for the cell, were also stained positively along their entire lengths. Their cytoplasm contained well-developed tubular structures corresponding to rough-surfaced endoplasmic reticulum and a small number of mitochondria, which were free from the S-100-like immunoreactivity. Immunopositive fibroblast-like cells were frequently found to hold bundles of collagen fibers. Immunonegative fibroblasts also occurred in the connective tissue. The negative fibroblasts possessed rich cytoplasm, being larger in size and possessing more mitochondria than immunopositive cells.

**DISCUSSION**

The present study demonstrated that the immunoreactivity for S-100 protein occurred in a cell population densely distributed in the connective tissue of the cardiac skeleton, valves and tendinous chordae. The S-100-positive cells appear to belong to the category of fibroblasts judging from their topographical and morphological features. However, typical fibroblasts in other regions of the heart and in other connective tissues of the entire body were not immunoreactive for S-100 protein. The immunopositive fibroblast-like cells in the heart were morphologically discriminated from immunonegative fibroblasts: their nuclei were rounder in shape; their cytoplasm was restricted to the perinuclear region; their cell processes were more slender like threads. The positive cells often appeared rounded in shape like chondrocytes.

We also recognized the S-100-positive cells in similar regions in the hearts of several mammals including the rat, dog, sheep, cow and man (MASANI, unpublished data). The existence of S-100 protein in the fibroblast-like cells of the heart seems to be common among mammalian species.

The occurrence of the immunoreactivity for S-100 protein in the cardiac fibroblast-like cells leads us to consider the relation of these particular cells to chondrocytes, which are known to be S-100 protein-positive cells. Earlier researchers have reported
on the presence of small cartilagenous nodules or bands in the heart of numerous ani-
mals including mammals, birds and reptiles (for review BENNINGHOFF, 1930). These
were found in the aortic ring and right fibrous trigone. The cartilaginous foci were
regarded as the results of a normoplastic transformation of fibrous tissue exposed to
special stress under physiologic conditions (HUEPER, 1939). These “ectopic” chondro-
cytes are likely to take their rise in the S-100 positive fibroblast-like cells. No reports
are yet available on the occurrence of cartilage or chondrocytes in the normal cardiac
valves, but the stroma of the valves resembles cartilage in its morphological aspects,
as pointed out by GREIL (1903). Under pathological and experimental conditions, the
occurrence of chondrocytes or cartilaginous tissue has been recognized in various parts
of the cardiac skeleton and valves more than in the aortic ring and right fibrous trigone:
the cardiac valves with endocarditis (ROSENSTEIN, 1900; SEEMAYER et al., 1973), the
cardiac skeleton which was injected with formalin (FISHEIN et al., 1977), or implanted
with bioprosthetic valves (ARBUSTINI et al., 1983).

Fig. 5. Proximal part of the mitral valve. Numerous S-100-positive cells are observed in the zona
spongiosa (upper half of the valvular stroma), while they are rare in the zona fibrosa (lower
half of the valvular stroma). Counterstain with hematoxylin. ×540

Fig. 6. Distal portion of the mitral valve. S-100-positive cells are distributed throughout the valve.
Endothelial cells are free from the immunoreactivity. ×340
Fig. 7. Mid portion of the mitral valve. Several Anitschkow cells (long arrows) in the zona spongiosa are weakly immunoreactive for S-100 protein. Arrowheads indicate immunonegative Anitschkow cells in the zona fibrosa. Counterstain with hematoxylin. ×850

Fig. 8. Electron micrograph of S-100-positive cells seen in the mitral valve. The immunoreaction is localized in their perinuclear cytoplasm and thread-like processes, along their entire length (arrows). A fibroblast with a wide cytoplasm is negative in reaction (on the right side of the figure). ×7,000
Cartilaginous tissues in the heart have been described as occurring in limited portions of the cardiac skeleton as mentioned above. In contrast, the present study demonstrated the zonal extension of S-100 positive fibroblast-like cells from the atrio-ventricular junctional area to the cardiac valves, and further to the tendinous chordae. This finding implies two possibilities: 1) The cardiac skeleton and valval stroma differ from the general connective tissues whose main role is to fill up the space between tissues. They need to be stout and flexible against the continuing strong movements of the heart. S-100 protein-containing fibroblast-like cells in the cardiac connective tissues appear to play an important role against dynamic stress. 2) The cardiac fibroblast-like cells in question in this study may be correlated with chondrocytes, both sharing a peculiar substance, the S-100 protein. Moreover, all the S-100 positive fibroblast-like cells in the cardiac skeleton and valves may have the potentiality to transform into chondrocytes.

In 1913, Anitschkow described certain cells with a peculiar nucleus, where chromatin accumulated in rod-shape, present among the granulation tissues experimentally formed in the myocardium (Anitschkow, 1913). These cells, called Anitschkow cells or caterpillar cells, also occurred frequently in inflammatory lesions of rheumatic myocarditis (Wagner and Stew, 1970), in cardiac metastases of cancer (Ragsdale, 1973) and in regenerative tissue following cold injury (Oron and Mandelberg, 1985). As reviewed by Pienaar and Price (1967), Anitschkow cells are scattered throughout normal cardiac tissues, though less numerously than in pathological hearts. The origin and significance of these cells remain in controversy. The present study revealed that a small number of Anitschkow cells in the cardiac skeleton and valves, not those in other parts of the heart, was also immunoreactive for S-100 protein, suggesting the relation between S-100 positive fibroblast-like cells and Anitschkow cells.

Recently, Kato and Kimura (1985) reported that S-100ao protein was primarily located in the heart and skeletal muscles of several mammals. This finding is in agreement with the present immunohistochemical study demonstrating the positive reaction for S-100 protein in the cardiac muscles, although the intensity of the immunoreactivity was considerably low. The weak reaction can be explained by the specificity of the antisera used, which were predominantly specific to the β subunit of S-100 protein.

Acknowledgements. The authors are grateful to Prof. Y. Takahashi, Department of Neuropharmacology, Brain Research Institute, Niigata University, for the generous gift the S-100 protein antisera and the antigen.

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