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Anti-rat Myoglobin Antisera in the Immunocytochemical Diagnosis of Rhabdomyosarcomas of Rats

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Abstract. Anti-rat myoglobin (Mb) was prepared and used in the avidin-biotin-peroxidase complex (ABC) method on paraffin-embedded sections of nine soft tissue tumors (including two rhabdomyosarcomas) of rats. Distribution and nature of the reactive substance to Mb antiserum were compared to those of desmin antiserum. Rat Mb was isolated from the skeletal muscle; monospecificity of the rat antiserum was confirmed by the immunoblotting procedures. The Mb antiserum reacted specifically to normal and neoplastic striated muscle cells. Mb-staining reactions were present diffusely in the cytoplasm, while desmin-staining substances were localized at Z-bands or were diffuse in the cytoplasm as separated aggregates. Reaction to the Mb serum was also detected in cells of thick portions of Henle's loop and distal convoluted tubules.

Most soft tissue sarcomas occurring in mice or rats are poorly differentiated and lack specific features of origin. These tumors have been categorized as small round cell, spindle cell sarcoma, or pleomorphic cell sarcoma, since conventional microscopic methods give only limited information which can be used to identify characteristics of the tumors.

Recently, immunohistochemical procedures have been successfully applied to the diagnosis and classification of soft tissue sarcomas in human pathology. Myoglobin (Mb) is known as a useful marker for the differentiation of rhabdomyosarcoma from other soft tissue tumors.[7,13,19] Antibodies to Mb available commercially are made from human skeletal muscle, and their application to laboratory animals seems practicable,[6,22] since antigenic cross reactivity exists to some extent among mammalian Mb.[3,10,15] However, there are fewer similarities between human and rodent Mb.[15] It would be preferable to provide an anti-rat Mb serum if an accurate diagnosis of rhabdomyosarcoma in rats is obtained.

In the present study we purified Mb from the skeletal muscle of the rats and prepared the antibody in the rabbits. The effectiveness of the rabbit anti-rat Mb serum in identifying rhabdomyosarcoma in the rats was assessed by the immunoperoxidase method. The distribution and nature of the reactive substance of the Mb antiserum in normal and tumor cells were compared to those of the anti-desmin serum obtained commercially. Desmin is well recognized as the constituent protein of intermediate filaments in muscle cells, and an anti-desmin serum is also used as a marker for muscle cells.[2,8,18]

Materials and Methods

Purification of rat Mb

Rat myoglobin (Mb) was purified from skeletal muscle according to the method of Luginbuhl,[17] with a few modifications. Ten Sprague-Dawley rats were killed by bleeding under ethyl ether anesthesia, and skeletal muscle was removed, frozen at −70 C, and stored until used. While still frozen, a sample weighing 63.3 g was chopped into small pieces and mixed in an electric blender with 5 ml of distilled water per gram of tissue. The mixture was adjusted to pH 8.1 with 2 N NaOH and stirred for 1 hour. Insoluble materials were removed by centrifugation at 16,000 × *g* for 30 minutes, and the supernatant was filtered through a coarse filter paper No. 2 (Toyo Roshi Co. Ltd., Japan).

An adequate amount of solid ammonium sulfate was added to the filtrate to obtain 20% saturated solution, and the mixture was stirred for 30 minutes and centrifuged at 16,000 × *g* for 30 minutes. More ammonium sulfate was added to the supernatant to increase concentration to 40% and then processed in the same way described above to obtain the second precipitate. The precipitating procedures were repeated through 60% and 80% saturated solution of ammonium sulfate. During the procedures, samples were kept at pH 8.1 by 2 N NaOH or 2% acetic acid solution. The supernatant obtained by centrifugation of 80% saturated solution was brought to 100% saturation by adding an excess amount of ammonium sulfate and allowing it to stand overnight, stirring constantly. It was then centrifuged at 39,000 × *g* for 40 minutes. The supernatant was adjusted to pH 6.8 with 2% acetic acid and kept at 4 C. Mb began to separate as white crystals after 4 days and was collected on a glass filter after 7 days.

Preparation of antiserum against rat Mb

Two female New Zealand albino rabbits were subcutaneously immunized by repeated injection of purified rat Mb

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over 14 weeks. About 380 μg of Mb protein emulsified in Freund's complete adjuvant (FCA) was injected at the dorsal region weekly for the first 3 weeks. Thereafter, the rabbits received a booster of the same doses together with Freund's incomplete adjuvant (FIA) biweekly for the next 8 weeks. During the period, blood samples were periodically withdrawn from the rabbits, and the reactivity of the antibody in the serum against rat Mb was checked on the paraffin-embedded sections of striated muscle cells of the rats. The serum from one rabbit exhibited a strong positive reaction to those cells even at a dilution of 1:400. This rabbit was bled 10 days after the last booster injection and serum was collected. The other rabbit that did not produce enough antibody available for immunohistochemical analysis was discarded without bleeding.

Furthermore, two female rabbits were prepared separately and were immunized with horse Mb (Biozyme Laboratories, Ltd., England) and given boosters on the same schedule as for rat Mb. The serum obtained from them was used as the standard to identify Mb protein on a gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done as described by Laemmli,[16] using a separating gel (12% acrylamide gel containing SDS) and a stacking gel (4% gel containing SDS). Samples obtained from precipitates of each step of fractionation with ammonium sulfate and Mb crystals were diluted two-fold in Tris-HCl buffer (pH 6.8) containing 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.002% bromophenol blue, and the mixture was boiled for 3 minutes. Electrophoresis was done for 3 hours at 60 V. The gels were stained with Coomassie brilliant blue.

Western blot analysis

Rat Mb or horse Mb antiserum obtained from rabbits was tested using Western blot analysis as described by Towbin et al.[23] Immediately after electrophoresis, the gel was set closely with a nitrocellulose membrane, immersed in Tris-glycine buffer, pH 8.3 containing methanol (20% vol/vol), and charged at 60 V for 3 hours. The protein in the gel was transferred to the nitrocellulose membrane. Thereafter, the membrane was incubated at 37 C for 60 minutes with appropriate dilution of the antiserum. Subsequently, the membrane was stained by the immunoperoxidase method that follows.

Immunohistochemistry

Avidin-biotin-peroxidase complex (ABC) method[12] was used on nine cases of tumors occurring in the subcutis of Wistar rats. The formalin-fixed specimen of a well-differentiated rhabdomyosarcoma (Case 1) was supplied by Dr. Y. Minato, Tanabe Seiyaku Company, Ltd., Osaka, Japan. Paraffin-embedded tissue from the remaining cases were selected from the files of the Pathological Division, Institute of Environmental Toxicology. Immunoenzyme staining was also applied to normal tissues from young rats including liver, kidney, spleen, heart, skeletal muscle, uterus, intestine, urinary bladder, sciatic nerve, and lung. These tissues were obtained from animals killed by bleeding. Tissues were immersed in 10% neutral buffered formalin (pH 7.4), retained for 1 to 4 weeks, and embedded in paraffin.

Identification of Mb or desmin was done as follows: 1) sections were deparaffinized in xylene and placed in absolute ethanol; 2) endogenous peroxidase activity was blocked with 0.6% H_2O_2 in methanol for 20 minutes; 3) to minimize non-specific reaction, sections were initially incubated with 20% calf serum in diluted oval albumin solution for 30 minutes at 37 C; 4) sections were then incubated with rabbit anti-rat Mb serum, rabbit anti-chicken desmin antibody (Dakopatts, USA), or normal rabbit serum (negative control) in 1:100 or 1:200 dilution overnight at 4 C and rinsed in phosphate-buffered saline (PBS); 5) sections were then incubated in biotinylated donkey anti-rabbit IgG antibody (Amersham, England) 1:200 for 30 minutes at 37 C; 6) after being rinsed again in PBS, sections were incubated with streptavidin-biotinylated peroxidase complex (Amersham, England) 1:200 for 30 minutes at 37 C; and 7) finally, sections were treated with 3,3′-diaminobenzidine-H_2O_2 mixture and counterstained with hematoxylin.

Results

Purification of rat myoglobin (Mb) and preparation of its antiserum in rabbits

Precipitates obtained by centrifugation of each supernatant of various degrees of saturation with ammonium sulfate were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was clear that the crude proteins with higher molecular weights than Mb were eliminated during the precipitating procedure of 20% to 80% saturated ammonium sulfate, and only Mb was left in the supernatant of 100% saturation. Purified rat Mb was a major band of approximately 17 kd corresponding to that of horse Mb (Fig. 1).

In Western blotting experiments, a clear single band was formed in the lane of the rat muscle homogenate after application of anti-rat Mb serum. The band was formed at the same level where the reactive product of horse Mb and its antiserum were demonstrated (Fig. 2). No positive band was detectable by normal rabbit serum. These results indicated that the antiserum to rat Mb was monospecific.

Immunohistochemistry

In the immunostaining of Mb to various normal tissues, cardiac and skeletal muscle fibers were strongly positive, although a few fibers were negative (Figs. 3, 4). Positive substances were demonstrated as fine granules distributed diffusely in the cytoplasm. Slightly positive reaction to anti-Mb serum was also seen in some erythrocytes, whereas smooth muscle fibers of the blood vessels, uterus, intestine, and urinary bladder were consistently negative. In the kidney, the tubular cells of thick portions of Henle's loop and distal convoluted tubules were stained with anti-rat Mb serum (Fig. 5).

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In the immunostaining of desmin, reactive substance in the striated muscle cells was localized mainly at Z-bands, and cross striations were remarkably accentuated (Fig. 4). Smooth muscle fibers in blood vessels, intestine, uterus, and urinary bladder were also positive for desmin.

Immunostaining using the antiserum against Mb or desmin was done on paraffin-embedded sections of soft tissue tumor suspected to be of myogenic origin from nine cases (Table 1). Of these cases, two were diagnosed finally as rhabdomyosarcoma on the basis of the results of the present immunohistochemical study in addition to conventional light microscopic findings. Case 1 was a well-differentiated rhabdomyosarcoma which was characterized by the presence of highly differentiated rhabdomyoblasts containing three or four nuclei and abundant eosinophilic cytoplasm (Type I) in which clear cross striations were sometimes seen (Figs. 6, 7). These cells were strongly positive for Mb (Fig. 6) but weakly positive for desmin (Fig. 7). In addition, there were numerous spindle-shaped cells with elongated nuclei and scanty eosinophilic cytoplasm (Type II) and a small number of round cells which were small in size with an oval, chromatin-rich nucleus and faintly basophilic cytoplasm resembling lymphoid cells (Type III). Type II and III cells did not disclose characteristics of the myogenic cells in sections stained with ordinary histological stainings. However, the immunostainings of Mb and desmin gave distinct positive reactions in most Type II cells (Figs. 6, 7), although Type III cells were consistently negative for these two markers. Case 2 was a poorly differentiated rhabdomyosarcoma consisting of Type II cells and a small number of Type III cells (Figs. 8, 9). No Type I cells were seen. The majority of Type II cells were positive for both Mb (Fig. 8) and desmin (Fig. 9). However, no positive reaction was found in Type III cells.

Distribution of the Mb and desmin in the tumor cells was markedly different. Mb-positive grains were found diffusely throughout the cytoplasm, while desmin-positive ones were seen as separated aggregates in the cytoplasm.

In other cases, no positive staining for these markers was found in any tumor cell, while degenerating muscle cells seen at the edge of tumors contained positive grains in the cytoplasm.

The specificity of immunostaining of Mb was confirmed; the positive reaction disappeared by replacing the antiserum with normal rabbit serum or by absorption of the antiserum with an excess amount of purified rat Mb.

Discussion

Myoglobin (Mb), an oxygen-binding heme protein of the skeletal and cardiac muscle, has been commonly used as a specific marker for neoplastic muscle cells, i.e., rhabdomyoblasts.[7,13,19] In several species of animals, Mb has also been purified and its antibody prepared.[1,14,20,21] The Luginbuhl method[17] used for purification of rat Mb in the present study was applied to the purification of human Mb and processed through ammonium sulfate solutions of various degrees of saturation kept at pH 8.0. The method was preferably applicable to rat Mb.

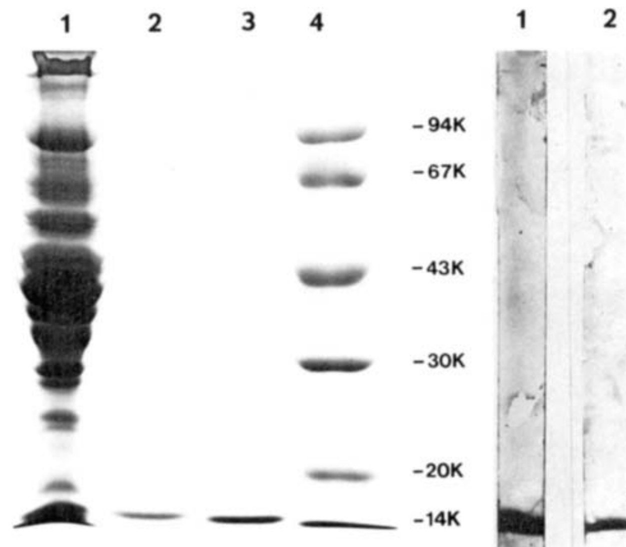

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified rat myoglobin (Mb). 1, Rat muscle homogenate (rMH); 2, purified rat Mb (rMb); 3, horse Mb (hMb); and 4, molecular weight markers.

Fig. 2. Western blot analysis of anti-rat myoglobin (Mb) antiserum. 1 (horse Mb; hMb) and 2 (rat muscle homogenate; rMH) were reacted with anti-horse Mb serum and anti-rat Mb serum. Avidin-biotin-peroxidase complex.

We have previously demonstrated the use of immunohistochemistry using anti-human Mb serum in the diagnosis of mouse rhabdomyosarcoma.[22] However, in such case the antibody raised against rodent Mb must react more specifically with neoplastic striated muscle cells than anti-human Mb antiserum, since the antigenicity of rodent Mb is less similar to that of human Mb.[15] According to enzyme-linked immunosorbent assay (ELISA) using rat Mb or human Mb as antigen, it was obvious that, as we had expected, the specific titer to rat Mb of anti-rat Mb serum (1 : 5,000) was higher than that of anti-human Mb serum (1 : 500), while the titer to human Mb of the former (1 : 1,000) was lower than that of the latter (1 : 10,000). Furthermore, when the specificity of immunostaining of anti-rat Mb serum was compared to that of anti-human Mb serum on the same paraffin-embedded sections of these rhabdomyosarcomas (Cases 1 and 2), the former surpassed the latter in the staining intensity of tumor cells, especially immature spindle-shaped myoblasts (Type

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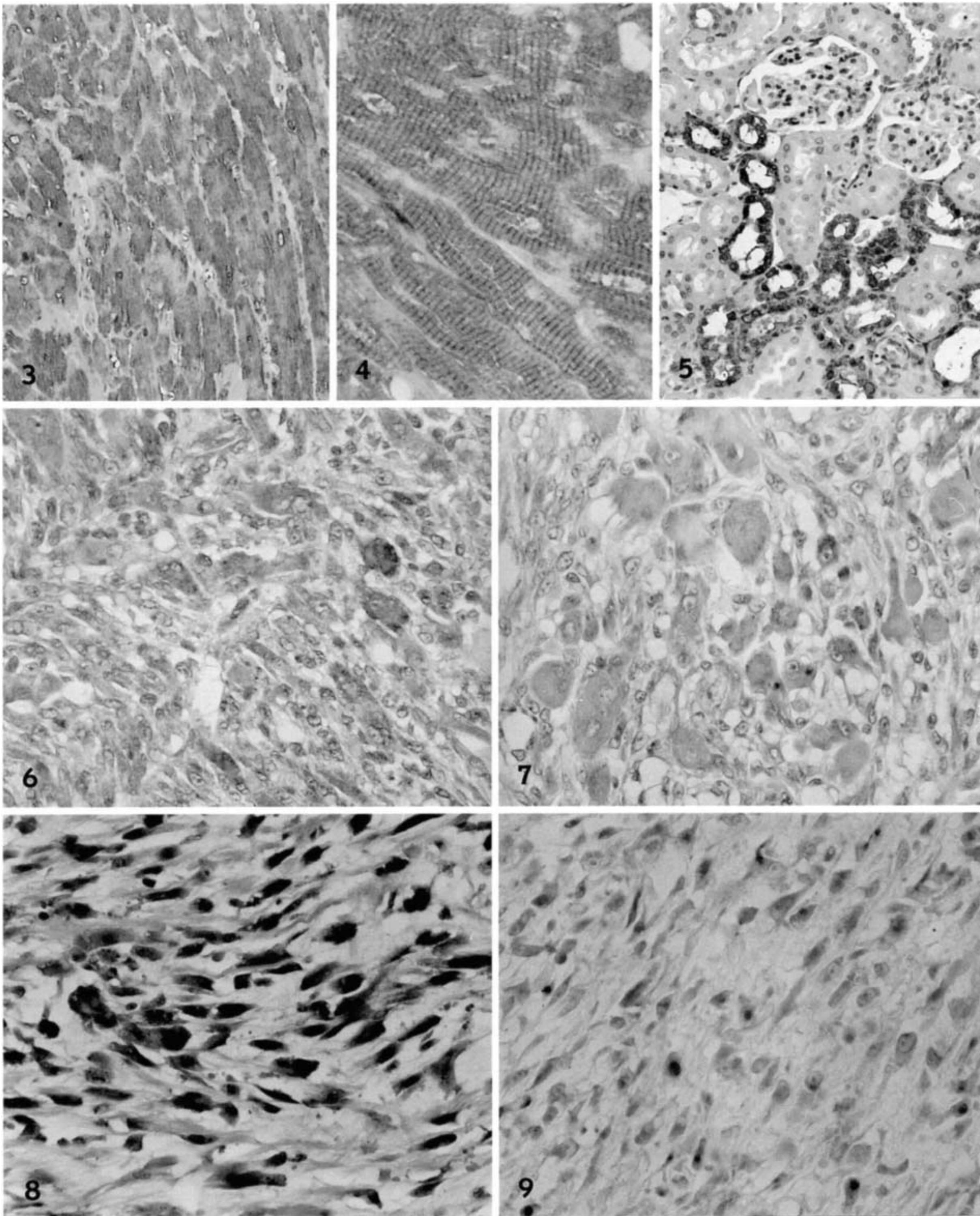

Fig. 3. Normal cardiac muscle. Diffuse distribution of myoglobin (Mb)-staining substance. Immunostaining of Mb.

Fig. 4. Normal cardiac muscle. Z-bands stain strongly for desmin. Immunostaining of desmin.

Fig. 5. Normal kidney. Distal tubular epithelia showing distinct myoglobin (Mb) reaction. Immunostaining of Mb.

Fig. 6. Well-differentiated rhabdomyosarcoma (Case 1). Multinucleated giant cells (Type I) and spindle cells (Type II) stain for myoglobin (Mb); small cells (Type III) are negative. Immunostaining of Mb.

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Table 1. Immunohistochemistry of soft tissue tumors in Wistar rats.

Case No.	Age (wk)/Sex	Site	Primary Diagnosis	Myoglobin (Mb)*	Desmin
1	5/M	Axillary region	Rhabdomyosarcoma	+	+
2	129/M	Hind limb	Spindle cell sarcoma	+	+
3	119/M	Hind limb	Pleomorphic cell sarcoma	−	−
4	104/F	Shoulder	Chondrosarcoma	−	−
5	36/M	Back region	Chondroblastoma	−	−
6	59/F	Abdominal region	Carcinosarcoma	−	−
7	90/F	Tail	Hemangiopericytoma	−	−
8	110/M	Hind limb	Fibrosarcoma	−	−
9	57/M	Thoracic region	Hemangiopericytoma	−	−

* + = positive; − = negative.

II) (K. Takahashi et al., personal communication). The difference in reactivity between these Mb antisera seems to depend upon different conformational determinants which are related to the three-dimensional structure of Mb.[3,4,10]

The present study showed a strong reaction in immature rhabdomyoblasts as well as multinucleated giant cells on paraffin-embedded tissues; the antiserum did not react with other tumors of non-muscle origin. Thus, the anti-rat Mb serum appears to be a useful tool to distinguish poorly differentiated rhabdomyosarcoma from other sarcomas in rats.

Tumor cells in two rhabdomyosarcomas stained intensely with both anti-Mb serum and anti-desmin serum. Distribution of the positive substances in the tumor cells differed: Mb-positive substances were present diffusely in the cytoplasm, in contrast with the localized distribution of desmin-positive substances. This may be partly due to differences in the intracellular location of each protein, since desmin is the main component of muscle-specific intermediate filaments, while Mb is the cytosol substance scattering throughout the cytoplasm.

Mb may be expressed only in the late phase in differentiation of the skeletal muscle cells,[14] while desmin is demonstrated in a relatively early phase.[2] In general, neoplastic transformation of striated muscle cells can be viewed as mimicking the condition during skeletal myogenesis.[9,11] During tumor development, neoplastic striated muscle cells may lose their ability to express some or all markers thought to be associated with their origin.[5,9] This concept is supported by our present results that Mb antiserum reacted more strongly with multinucleated rhabdomyoblasts (Type I) than immature spindle-shaped myoblasts (Type II). The desmin antiserum demonstrated only weakly positive reaction in Type I cells, whereas it gave a strong response in Type III cells, as did Mb antiserum. The small round cells (Type II) resembling primitive mesenchymal cells reacted with neither of them. The heterogeneity of these tumor cells may reflect different levels of myogenic differentiation.

The staining reaction in some erythrocytes was probably due to incomplete blocking of endogenous peroxidase[6] since such reaction was not eliminated by replacing Mb antiserum with normal rabbit serum. The positive reaction for anti-Mb serum in renal tubules may be due to antigenic cross-reaction between rat Mb molecule and other components within these epithelial cells.

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Fig. 7. Well-differentiated rhabdomyosarcoma (Case 1). Staining for desmin is stronger in spindle cells than multinucleated giant cells. Small cells do not stain. Immunostaining of desmin.

Fig. 8. Poorly differentiated rhabdomyosarcoma (Case 2). Most spindle cells are strikingly positive for myoglobin (Mb). Immunostaining of Mb.

Fig. 9. Poorly differentiated rhabdomyosarcoma (Case 2). Desmin-positive reaction partly in cytoplasms of spindle cells. Immunostaining of desmin.

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