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# Distribution of β-enolase in normal and tumor rat cells

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**Abstract:** Enolase – a glycolytic enzyme is also expressed on the surface of eukaryotic cells such as macrophages, neutrophils, endothelial, neuronal, tumor cells. Surface enolase as plasminogen receptor plays an important role in myogenesis, tumorgenesis and angiogenesis. Determination of enolase localization in the cell lines may give rise to the elucidation of its receptor function in tumor cells. The cellular localization of the muscle-specific isoform of the enolase in normal rat cardiomyocytes (H9c2, an embryonic rat heart-derived cell line) and a rat sarcoma (R1) cell line is reported here. Immunocytochemical assays showed that this enolase isoform is freely diffused in the sarcoplasm of rat cells. The evident location of enolase molecules on the perinuclear surface is observed in immunofluorescence assays. Enolase localization on the surface of some intact normal rat cardiomyocytes was also observed. This surface protein maintains enolase catalytic activity.

**Key words:** beta-enolase, cellular localization, rat cardiomyocytes, rat sarcoma

## Introduction

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) is a glycolytic enzyme in the cell's cytosol compartment of all organisms metabolizing glucose along the Embden-Meyerhoff-Parnas pathway. In higher vertebrates, enolase is active as a dimer formed from three different subunits α, β and γ, which are coded by separate genes. The expression of these genes are developmentally regulated in a tissue-specific manner. The ubiquitous αα isoform remains widely distributed in embryonic most adult cell types. In adult muscle tissues, the β-enolase subunit accumulates preferentially in fast-twitch fibres, where the ββ homodimer accounts for more than 90% of the total enolase activity [1].

Many reports are available demonstrating the direct correlation between increased expression of enolase and progression of neuroendocrine tumors, neuroblastoma and lung cancer [2]. The expression level of enolase is too high for its contribution to glycolysis alone [3]. On the other hand, enolase is recognized as a multifunctional protein. It is ubiquitously located in the cytoplasm as a glycolytic enzyme, but also identified on the surfaces of many eukaryotic and prokaryotic cells, where it plays a role as a receptor of some ligands. Numerous data present enolase as an effective plasminogen receptor [4]. In this way enolase may arm various cells with proteolytic properties and facilitate tissue growth by the degradation of extracellular matrix (ECM). The degradation of extracellular matrix proteins in this system is very important in the spread of tumor cells [5,6], in myogenesis and in the process of muscle tissue remodeling after injury [7,8].

In the present report we demonstrate the localization of enolase in normal (H9c2) and transformed rat muscle cells (the rat rhabdomyosarcoma R1 line) by fluorescence microscopy and immunocytochemical analysis using rabbit antibodies specific against β-enolase from human striated muscle. Determining enolase localization in these cells may give the rise to further study of its receptor function in tumor cells.

## Materials and methods

**Cell cultures.** The rat sarcoma (R1) cell line was obtained from the Institute of Immunology and Experimental Therapy of the Polish

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**Abbreviations:** ERK, extracellular-signal-regulated kinase; MEK/ERK kinase; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; TRITC, rhodamine isothiyocyanate;


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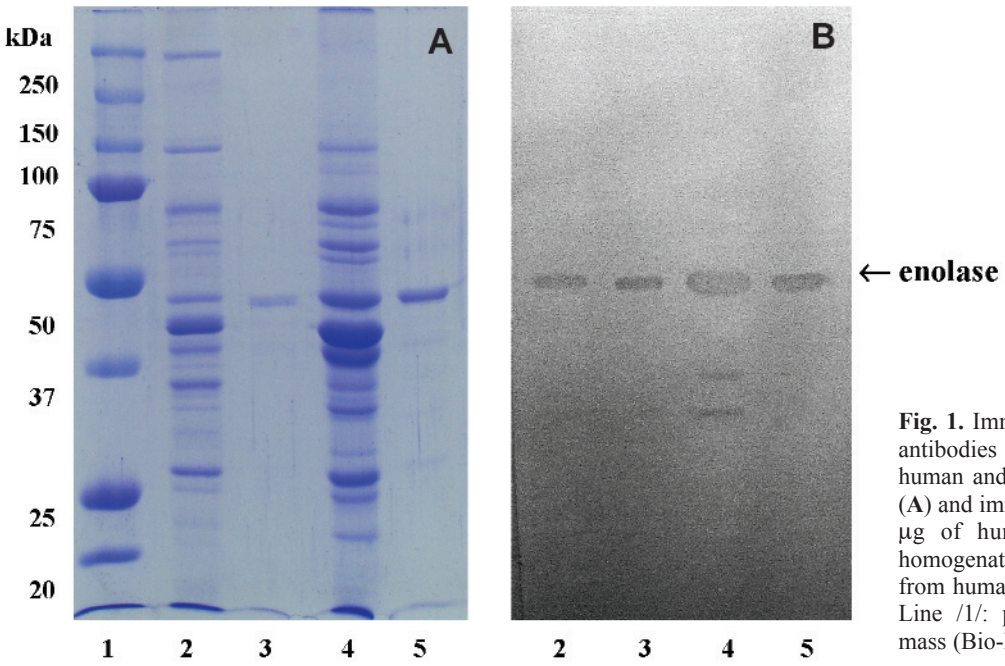


**Fig. 1.** Immunereactivity of rabbit specific antibodies against human β-enolase with human and rat enzyme – 10% SDS/PAGE (**A**) and immunoblotting (**B**): samples of 25 μg of human /2/ and rat muscles /4/ homogenates and 2 μg of purified β-enolase from human /3/ and rat /5/ striated muscle. Line /1/: protein standards of molecular mass (Bio-Rad).

Academy Sciences (Wroclaw, Poland) and normal rat cardiomyocytes (the embryonic rat heart-derived cell line H9c2) were a gift from prof. F. Ursini from University of Padova (Italy). The cells were grown in DMEM medium (Sigma) with 3% glutamine, 10% foetal calf serum and antibiotics: gentamycin (100 μg/ml) and penicillin (100 U/ml). The cell lines were incubated at 37°C with 5% $CO_2$.

**Antibodies against human β-enolase**. Human tibialis anterior muscle was obtained from postoperative material from the Department of Vascular, General and Transplantation Surgery of the Wroclaw Medical University in accordance with the Polish legal requirements, under a license issued by the Commission of Bioethics of the Wroclaw Medical University.

Purification of enolase from the human striated muscle, preparation of rabbit polyclonal serum against human β-enolase and isolation of specific antibodies from the rabbit serum by affinity chromatography were preformed according to our previous report [9]. The specificity of the obtained antibodies was confirmed on homogenates of human and rat striated muscles and β-enolase purified from human and rat tissue. Gel electrophoresis and immunoblotting were performed under the conditions previously described [9].

**Immunocytochemical staining**. Immunocytochemical reactions were performed using the avidin biotinylated peroxidase (ABC) technique (DAKO LSAB2 System HRP, Denmark). Specific polyclonal rabbit antibodies against human β-enolase were applied.

**Cell surface enolase activity**. Enolase activity of intact normal and tumor cells was determined by a direct assay [10].The cell stock solutions contained $6.73\times10^5$ H9c2 cells and $12.8\times10^5$ R1 cells were resuspended in 50 mM imidazole pH 6.8 buffer containing 10 mM $MgSO_4$ and 10 mM KCl and subsequent dilutions (1:1; 1:2; 1:4; 1:8 and 1:16 v/v) were used to determined of surface enolase activity.

The enolase substrate 2-PGA was added to the reaction mixture in a 6 mM concentration and conversion of the substrate to phosphoenolpyruvate (PEP) was followed at 37°C for 3 min. The cells were removed by centrifugation (3000 rpm for 1 min). The level of PEP production of the supernatant was measured at 240 nm. In the same manner enolase activity was detected in the normal and tumor rat cells under thermal and osmotic shock conditions.

**Immunofluorescence studies**. All cell cultures were suspended in PBS with Triton X-100 (Sigma). To stain β-enolase, the cells were incubated first with rabbit polyclonal antibodies against β-enolase (1:1000 v/v) and then with rhodamine isothiocyanate-labeled mouse anti-rabbit IgG (Sigma). The control for specificity involved omission of the primary antibodies and the cells were incubated only with 2% BSA. The specimens were evaluated using fluorescence microscope (Zeiss, Germany).

**Statistical analysis**. Experiments were performed in triplicates. Data for activity of surface enolase were analyzed by Student's t-test. The mean value was presented on figures.

## Results

Enolase immunolocalization was performed on the normal and sarcoma rat cells. For this study the antibodies were affinity-purified from rabbit polyclonal antiserum on a Sepharose CL-4B column with immobilized human muscle enolase as described previously [9]. Their specificity was confirmed by immunoblotting with homogenates of human and rat normal striated muscle and pure enolase from both tissues (Fig. 1). Rabbit antibody produced against human β-enolase reacts also with rat muscle enolase. These antibodies we applied to detect β-enolase reactivity in the sarcoplasmatic compartment of normal rat cells (H9c2) by immunocytochemical staining. Most of the enolase molecules were diffused throughout the sarcoplasm (Fig. 2).

The distribution of enolase in the normal rat and rat sarcoma cells were observed by immunofluorescence microscopy (Figs 3 and 4). We observed different size


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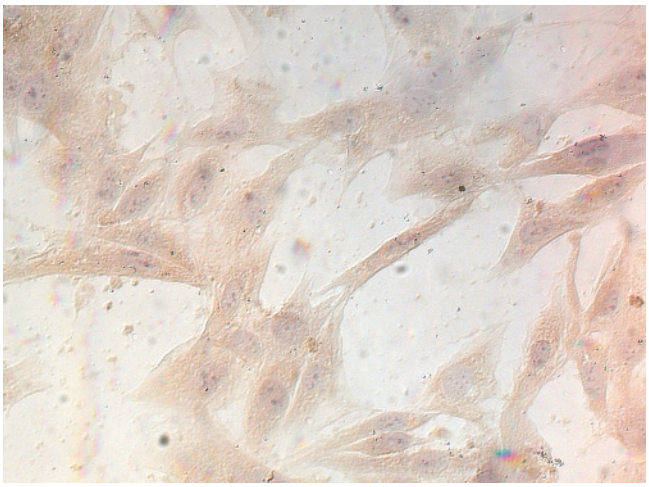

**Fig. 2.** Immunocytochemical detection of β-enolase in rat normal cardiomyocytes (the embryonic rat heart-derived cell line H9c2) using rabbit anti-human β-enolase antibodies (magnification, ×400).

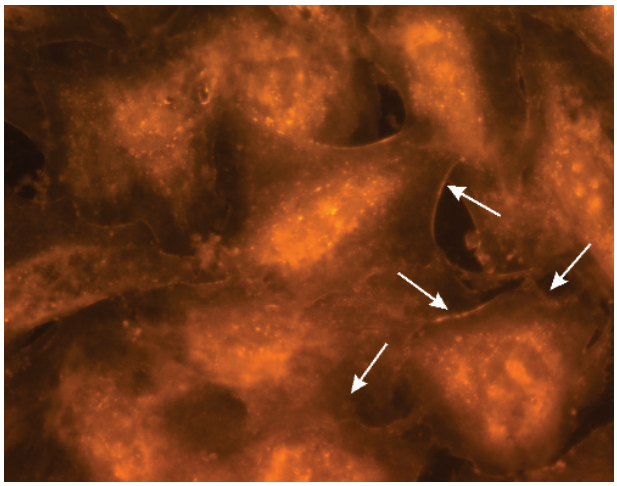

**Fig. 3.** Immunofluorescence detection of enolase in normal rat cardiomyocytes (H9c2) incubated with rabbit anti-human β-enolase antibodies (magnification, ×400). Arrows indicate cytoplasmic and surface localization of enolase.

areas of fluorescence-labeled proteins in the cytoplasm of the normal, and sarcoma cells. Clearly visible fluorescence of the cytosol compartment in both cell lines and less expressed fluorescence on the surfaces of some cells were observed. Furthermore, a more intensive fluorescence of β-enolase near the perinuclear surface of rat sarcoma sarcoplasm was noted in comparison with normal rat cardiomyocytes (Figs. 4 and 3, respectively).

We found enolase catalytic activity in intact muscle cells of normal rat cardiomyocytes as well as in the culture of rat tumor cells (Fig. 5 A,B). A dose-dependent enolase activity *i.a.* 2-PGA conversion to PEP in serially diluted intact cells was noted. In the absence of 2-PGA the intact cells did not reveal any enzymatic activity and were used as a negative control. We used log-phase grown cell cultures to rule out the possibility that enolase activity was not due to enzyme released as a result of cell lysis. In additional experiments we compared the production of PEP in normal and sarcoma rat cells under osmotic and thermal shock conditions (Fig. 5 A,B). The lysis of rat cells caused under these conditions revealed higher PEP levels in the assay medium compared with experiments with intact cells.

Some differences between the surface enolase activities of normal rat cardiomyocytes and tumor cells were observed. The $12.8\times10^5$ cells from the rat sarcoma culture used in the experiment demonstrated a significantly lower ($0.230\pm0.02$ vs. $0.330\pm0.04$, $p=0.016$) enolase activity than $6.73 \times 10^5$ normal cells. This suggests weak availability to the substrate or low level of surface enolase expression on the rat tumor cells used in the experiments.

## Discussion

β-enolase is expressed in the striated muscle of mammals and humans. In the present study we demonstrated the distribution of muscle-specific enolase in nor-

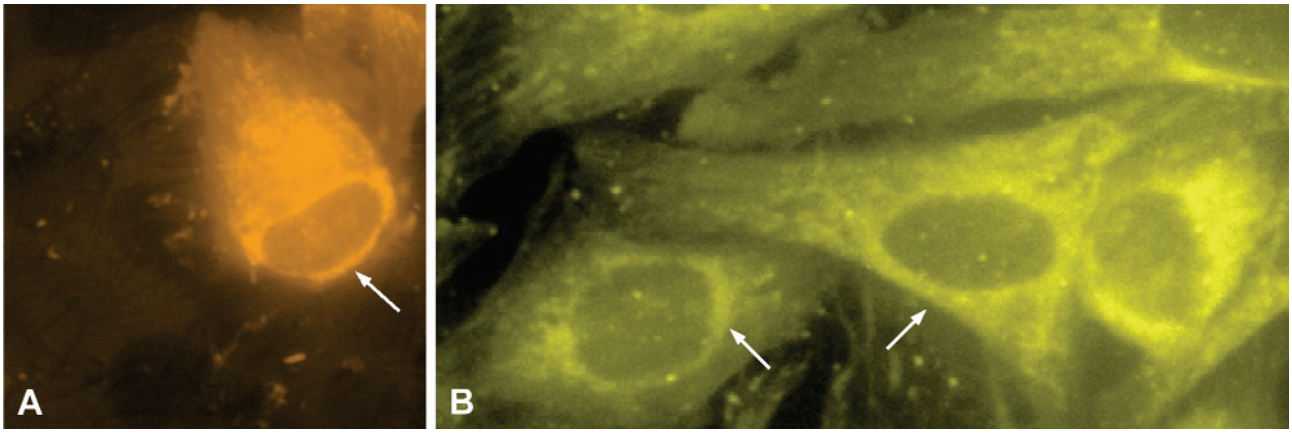


**Fig. 4.** Immunofluorescence detection of enolase in a rat sarcoma cell line (R1) incubated with rabbit anti-human β-enolase antibodies (magnification, x 400). Arrows indicate of surface (**A**) and intracellular localization (**B**) of enolase.


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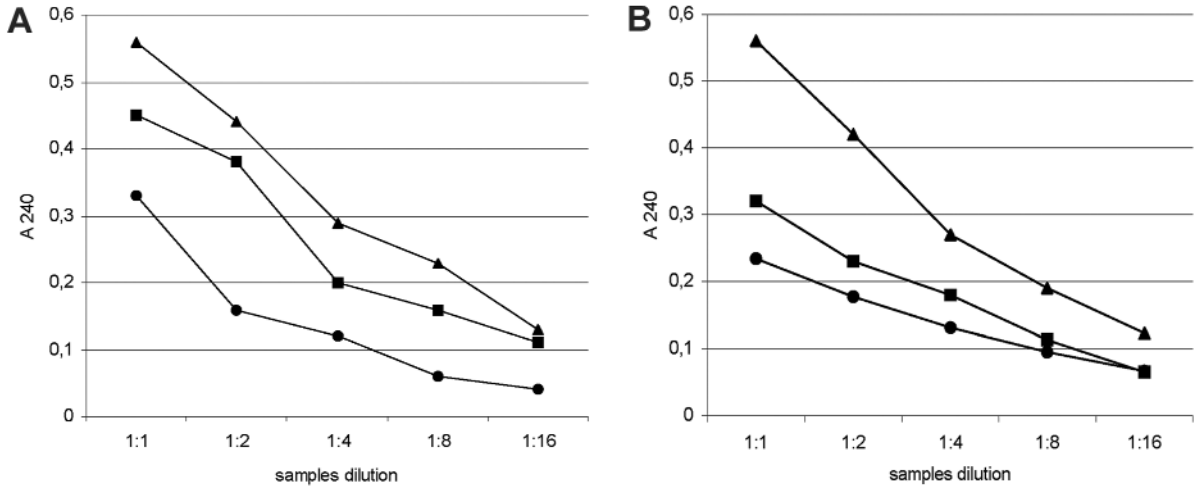


**Fig. 5.** Detection of surface enolase catalytic activity in normal intact rat muscle cells (A) and rat sarcoma cells (B). The experiment was performed in: (●) intact cells in 50 mM imidazole pH=6.8 with 10 mM $MgSO_4$ and 10 mm KCl, (■) under osmotic shock: cells incubated for 3-4 h at 25°C in 10 mM imidazole, pH=6.8 with 2 mM $MgSO_4$, 1.5 mM KCl and protease inhibitors (Minicomplete ROCHE), (▲) under thermal shock: cells suspended in 50 mM imidazole, pH=6.8 with 10 mM $MgSO_4$ and 10 mM KCl and protease inhibitors (Minicomplete ROCHE), twice frozen at -80°C and thawed. Three independent experiments were performed.

mal rat and rat tumor cells. We used the clonal rat muscle cell line H9c2 as a cell model of normal cardiac myocytes. This cell line is derived from embryonic rat heart tissue and has properties of skeletal muscle as well as cardiac muscle [11,12].

We have used rabbit antibodies against human β-enolase in our experiments. The positive immunoreaction given by normal rat cardiomyocytes and rat sarcoma cells with rabbit anti-human β-enolase antibodies suggests the similarity of some epitopes of muscle-specific enzyme in both species. Similar cross-reactivity between human muscle enolase and antibodies against the mouse β-enolase was observed by other authors [13]. This is not surprising because enolase is known to be an evolutionarily highly conserved protein [14]. An over 80% identity in amino-acid sequence between human and rat β-enolase monomers was noted [4,13].

In immunocytochemical analysis we observed a positive reaction of β-enolase in normal rat cardiomyocytes. This protein was diffused throughout the sarcoplasmatic compartment. Our results correspond to those reported by other authors, who found β-enolase immunoreactivity throughout the sarcoplasm of mouse striated muscle cells [15]. The mobility of globular proteins in cytoplasm depends on their relative diffusion coefficient and molecular size. These parameters estimated for enolase in a rabbit muscle cell culture indicate a relative high intersarcoplasm diffusion [16].

In rat and mouse skeletal muscle, total enolase activity is highest in fast-twitch muscle, and this activity is almost entirely due to the muscle-specific homodimer ββ. However, in rat cardiac muscle which exhibits a lower enolase activity, the three isozymes αα, αβ and ββ are almost equally abundant [17]. It is most likely that the immunoreactivity observed in our experiments in rat cells is related to two isoforms, the heterodimer αβ and the homodimer ββ. The higher level of surface enolase catalytic activity on the H9c2 intact cells in comparison to R1 cells can result from embrional source of this line of rat cardiomyocytes and suggest the potentially important role of this protein in developmental growth of heart tissue.

According to our data, enolase is present on the surface of intact rat cardiomyocytes and sarcoma cell lines and maintains catalytic activity. The ability to convert 2-PGA to PEP by intact cells suggests that the surface protein keeps the native conformation. A similar localization and catalytic activity of enolase in synaptic membranes of rat neurons was detected [18]. Our experiments with intact cells were performed under conditions avoiding cell lysis. The significant increase in enolase catalytic activity observed in normal rat and rat tumor cells after osmotic and thermal shock is due to cell lysis.

We detected enolase activity on surface of rat sarcoma cells although we did not observe evident fluorescence of surface enolase on rat tumor cells. However, some of the normal rat cardiomyocytes demonstrated surface fluorescence. According to recent reports, the presence of plasminogen in culture medium stimulates enolase overexpression by the MEK/ERK pathway [19]. The cell culture in our experiments was performed without plasminogen. Therefore, the lack the fluorescence of the rat sarcoma cell surface and weak fluorescence only on normal cells probably resulted from


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another, unknown mechanism regulating the enolase surface expression. No known muscle enolases possess a signaling sequence, which may act as a sequence targeting the protein to the plasma membrane [20].

We detected an evident accumulation of rat muscle enolase molecules near the nuclear surface in both cell lines. We observed that the perinuclear immunoreactivity of β-enolase in the rat sarcoma cells was more intensive than in normal rat cells. This corresponds to other reports on the localization of mouse muscle enolase in regenerating tissue [1]. However other authors report a nuclear location of enolase molecules in rat neurons [21] and HeLa cells [22]. Enolase was shown to be a nuclear factor negatively regulating c-myc promoter activity. c-Myc protooncogene plays an important role in the regulation of cell growth and differentiation. Overexpression of c-myc gene is a common characteristic feature of many malignant cell types [23]. Some other enzymes whose primary described function is participation in the glycolytic pathway in the cytoplasm have so far been found in nuclei. Their physiological roles in the nucleus seem to be different from those in the cytosol compartment. Phosphoglycerate kinase is supposed to participate in DNA synthesis and cell cycle progression [24]. Glyceraldehyde-3-phosphate dehydrogenase recognizes the sequence and structural features of RNA and is involved in DNA replication and repair [25]. Lactate dehydrogenase has been found to be a stabilizing nuclear factor participating in DNA repair [24].

According to recent reports, the expression of enolase is too high to be justified by its contribution to glycolysis alone [3,4] and may explain the presence of enolase on the surface of some intact normal rat cardiomyocytes and rat sarcoma cell lines. Surface enolase exposition on myogenic cells during muscle development has also been observed [26]. This may suggest that muscle-specific enolase can exhibit functions unrelated to its pivotal role in the sarcoplasm as a glycolytic enzyme. Surface enolase was recently recognized to be a receptor for plasminogen [27]. The binding of plasminogen and its subsequent pericellular activation is an important process in the development of normal muscle tissue [26] in remodeling after injury [7] and in tumor growth [6,28]. The influence of some effectors on the interaction between plasminogen and surface enolase in selected cell cultures should be examined in the future in more detail.

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