Possible Involvement of Hic-5, a Focal Adhesion Protein, in the Differentiation of C2C12 Myoblasts

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ABSTRACT. Hic-5, a focal adhesion protein, has been implicated in cellular senescence and differentiation. In this study, we examined its involvement in myogenic differentiation. The $\text{hic-5}$ expression level in growing C2C12 myoblasts increased slightly on the first day and then gradually decreased until no $\text{hic-5}$ was detectable after 7 days of differentiation. In vivo, its expression level declined in the thigh and the calf skeletal muscle of mouse embryos after birth. The introduction of an antisense expression vector of $\text{hic-5}$ into C2C12 cells decreased the number of clones expressing the myosin heavy chain (MHC) upon exposure to the differentiation medium. In the cloned cells with low levels of $\text{hic-5}$, the efficiency of myotube formation was significantly reduced. The expression levels of MyoD, myogenin, MHC and p21 were also reduced in these clones. The results suggested that $\text{hic-5}$ plays a role in the initial stage of myogenic differentiation.

Key words: Hic-5/C2C12/myogenic differentiation

Although lineage-specific transcription factors like MyoD, Myf5, myogenin and MEF2 play central roles in the differentiation of cells of the myogenic lineage (Arnold and Winter, 1998), there is a body of evidence that points to the importance of the cellular microenvironment, representative of which are cell-extracellular matrix (ECM) interactions during embryonic development and cellular differentiation, including myogenesis (Adams and Watt, 1993; Gumbier, 1996). For example, laminin-1 plays a prominent role in promoting myotube formation along with adhesion and proliferation of myoblasts, while fibronectin inhibits myogenic differentiation (von der Mark and Ocalan, 1989). Integrins at the cell surface are thought to mediate the signals of the cell-extracellular matrix interactions, regulating development and cellular differentiation as well as cell shape, motility, and growth (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Fassler et al., 1996). Menko and Boettiger demonstrated that the interaction of integrin with ECM was essential to initiate the terminal stages of myogenic differentiation (Menko and Boettiger, 1987). $\text{hic-5}$ was first isolated as TGFβ1- and hydrogen peroxide-inducible cDNA from mouse osteoblastic cells (Shibanuma et al., 1994). Structurally, it possesses four LIM and three LD domains, both of which potentially serve as interfaces for protein-protein interactions, in its C- and N-terminal regions, respectively, and shows striking similarity to paxillin (Turner, 2000; Thomas et al., 1999). Its localization at focal adhesions and structural features suggested that Hic-5 plays some role as an adaptor molecule in integrin signaling through interaction with several kinds of signaling molecules (Nishiya et al., 2001), thereby modulating cell phenotypes. In fact it has been implicated in the cellular senescence of fibroblasts and differentiation of osteobasts (Shibanuma et al., 1997; Shibanuma and Nose, 1998; Ishino et al., 2000). Its association with platelet development was also reported by Hagmann et al. (Hagmann et al., 1998), although the molecular mechanisms underlying its biological effects are still largely unknown. The molecular aspects and biological effects of Hic-5 as described above suggested its involvement in broad range of phenotypic change of cells that were associated with integrin signal. We here examined its involvement in the differentiation of C2C12 myoblasts, where cell-ECM interactions and/or integrin signals are supposed to play important roles in modulating the phenotypes as mentioned above.

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Abbreviations: MHC, myosin heavy chain; MLC, myosin light chain; ECM, extracellular matrix; GD, glyceraldehyde-3-phosphate dehydrogenase.
Materials and Methods

Cell culture

Mouse myoblastic cells, C2C12, were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 15% fetal bovine serum and 50 µg/ml kanamycin under a humidified atmosphere of 5% CO₂ in air. To induce the differentiation, the medium was replaced with the differentiation medium containing 2% horse serum instead of 15% fetal bovine serum.

Northern blots

Total RNA was extracted, purified and Northern blotted essentially as described in Shibanuma et al., 1997. The probes for hic-5 and glyceraldehyde-3-phosphate dehydrogenase (GD) were the same as in Shibanuma et al., 1997. Mouse cDNA fragments of MyoD, myogenin, myosin heavy chain (MHC) and myosin light chain (MLC) provided by Dr. A. Asakura (Fred Hutchinson Cancer Research Center, Seattle) and of p21 provided by Dr. B. Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MA) were used as probes. The radioactivity was quantified with a Bioimage Analyzer (Fuji Photo Film, Tokyo). The values were normalized to that of GD as a monitor of the amount of RNA in each lane.

Expression plasmids, transfection and analysis of clones

Expression vectors, CMV/S5 (Shibanuma et al., 1997) for sense and pEF-BOS-anti-hic-5 (Shibanuma and Nose, 1998) for antisense expression of hic-5, were introduced into C2C12 cells together with pSV2neo by the conventional calcium phosphate coprecipitation method (Graham and van der Eb, 1973), and the cells were selected with G418 (400 µg/ml) for 10 days. As a control plasmid, the empty vector, pRc/CMV or pEF-BOS, was used for transfection. The colonies were then placed in the differentiation medium for 8 to 12 days to analyze MHC expression or picked up and expanded for clonal analysis. Western blotting was performed as described previously (Ishino et al., 2000).

Immunostaining for MHC

The colonies were first treated with 0.1% saponin for 15 sec, fixed with 3.7% formalin for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min as previously reported (Arber et al., 1994). Subsequent processes were done as described previously (Ishino et al., 2000). The first antibody used was monoclonal anti-skeletal myosin M-4276 (Sigma Chemical Co., St. Louis, MO), and the second antibody was anti-mouse IgG peroxidase-labeled (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD). The immunocomplex was visualized with a DAB Substrate Kit (Vector Laboratories, Inc., Burlingame, CA).

Mouse embryos

Pregnant ICR mice at 12 days of gestation were purchased from the Saitama Experimental Animals, Co., Saitama.

Results and Discussion

We first examined expression patterns of hic-5 during myogenic differentiation in vitro. Logarithmically growing C2C12 cells were exposed to a low mitogen medium containing 2% horse serum to cause them to differentiate. After a given period, total RNA was extracted and Northern blotted with probes for hic-5 along with those for MyoD, myogenin, myosin heavy chain (MHC) and myosin light chain (MLC). MyoD and myogenin are basic helix-loop-helix transcription factors controlling skeletal muscle development at the initial stage (Arnold and Winter, 1998). MHC and MLC are proteins expressed typically in differentiated skeletal muscle cells and used as differentiation markers of the myogenic lineage. Figure 1 shows that the expression level of hic-5, among the above mRNAs, was the first to change; a high level of expression was detected in the proliferating myoblasts and the expression increased slightly on the first day after the switch. It then decreased and became hardly visible after 7 days. Changes of MyoD and myogenin expression followed those of hic-5 expression; their levels increased with a peak on the second day and then decreased. Unlike these transcripts, MHC and MLC, which were undetectable early in the process, began to be expressed after 2 days, followed by the formation of myotubes. Myotubes were visible 6 days after the switch (data not shown).

A similar change in the expression pattern of hic-5 was observed in vivo during development of the skeletal muscle. As shown in Fig. 2, hic-5 mRNA was highly expressed in the thigh and the calf muscle from embryonic day 16 to 18. After birth, its expression decreased and became barely detectable in the adult muscle. From these expression patterns, hic-5 was speculated to play some role in the regulation of the terminal differentiation of myoblasts at an early stage in the process.

To test the above possibility directly, we transfected sense (CMV/S5) and antisense (pEF-BOS-anti-hic-5) expression vectors of hic-5 carrying a neomycin resistance gene into C2C12 cells and assessed the effects of their expression on the C2C12 differentiation (Fig. 3). Following selection with G418 for 10 days, colonies were placed in the differentiation medium with low serum for 8 or 12 days. The density of the colonies was controlled at about 50 to 100 colonies/90 mm dish, to prevent the enhancement of myotube formation due to cell-cell interactions. As a differentiation marker, the expression of MHC was evaluated by immunostaining, and colonies positively stained with the antibody to MHC were enumerated. The sense expression affected marginally the number of MHC-positive colonies (Fig. 3A). The overexpression of hic-5, thus, might lead to neither enhancement nor suppression of MHC expression. Alternatively, the cells that overexpressed hic-5 would selectively die of apoptosis, making it impossible to evaluate the effects of overexpressing hic-5 in this kind of assay. Taken the re-
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cent report by Hu et al. showing that overexpression of hic-5 resulted in the inhibition of myogenesis by inducing apoptosis (Hu et al., 1999), we favored the latter possibility. Consistent with this idea, no clones expressing hic-5 over 2-fold to the parental cells were obtained after screening more than 50 clones in our trial to establish a stable cell line overexpressing hic-5. In contrast to the sense expression, the antisense expression evidently reduced the number of colonies expressing MHC (Fig. 3B). This observation suggested that expression of hic-5 above a certain level was required for efficient induction of MHC.

The involvement of hic-5 in the myogenic differentiation was further examined in detail by isolating and analyzing each of the antisense-transfected colonies; the G418-resistant colonies obtained in the antisense-transfected populations as above were isolated, expanded, and induced to differentiate. As shown in Fig. 4A, the hic-5 protein levels of the clones varied from 0.63 to 1.1 of that in the parental C2C12 population. Although the difference in the level of hic-5 expression among the clones was modest, the hic-5 expression level was well correlated with the efficiency of myotube formation among 6 independent clones examined; as shown in Fig. 4B, the clones expressing almost the same level of Hic-5 as parental cells, such as #5 and #9, had well-developed myotubes and so did to a lesser extent, clone #10, being consistent with their hic-5 expression level of 1.0, 1.1, and 0.85, respectively. However, the myotube formation was significantly impaired in clones such as #22 and #B4 whose hic-5 expression levels were reduced to 0.63 or 0.73. Consistent with this, MHC mRNA expression was also remarkably suppressed in these clones (Fig. 5A).

The core regulatory network for the myogenic differentiation has been identified at a molecular level, and a genetic hierarchy has been revealed among the molecules controlling skeletal muscle development (Arnold and Winter, 1998); MyoD or Myf5 specifies cells to adopt the myoblast fate and then, in response to a trigger of the differentiation, the expression of myogenin and p21, which are required to control the differentiation process directly and cell-cycle arrest (Parker et al., 1995; Zhang et al., 1999), respectively, is induced. Following these molecular events, muscle-specific proteins are produced, such as MHC and MLC. On the basis of this model, we examined the mRNA levels of MyoD and myogenin among the above clones and tried to obtain some
insight into the stage of differentiation at which hic-5 was involved. In clones #B4 and #22 whose expression levels of hic-5 were relatively low, the expression of myogenin as well as MHC was significantly repressed (Fig. 5A). MyoD expression levels were also reduced in these clones, though to a lesser extent, upon exposure to the differentiation medium (Fig. 5A, lanes 2 to 4 labeled by MyoD); although in the proliferating state, the average level of MyoD expression in the low-expressing clones was comparable (0.83) to that in the parental cells (Fig. 5A, lane 1 labeled by MyoD), the level was reduced to 0.44 of that in the parental cells in the differentiating state. To determine whether the decrease in MyoD in the differentiating #B4 and #22 cells was biologically significant or not, we examined p21 expression levels in the clones. According to a previous report, MyoD was a positive regulator of the up-regulation of p21 (Guo et al., 1995; Halevy et al., 1995). In the present experiments, the induction of p21 expression was found to be attenuated in #B4 and #22, compared to that in the other clones expressing hic-5 at the level comparable to the parental cells, as expected (Fig. 5). These results suggested that hic-5 was primarily required for the expression of MyoD and myogenin at the initial stage of the differentiation shortly after cell commitment, maintaining the potential of myoblasts to differentiate and/or preventing them from apoptosis. The clones studied here all exhibited low apoptotic index in the differentiation condition, which hindered our analysis of the effects of Hic-5 on apoptosis (data not shown). In conclusion, it was suggested that low-level expression of hic-5 below a critical threshold resulted in the blockage of a cascade of the molecular events subsequent to the MyoD and myogenin expression. The expression pattern of hic-5 described above was consistent with this hypothesis.

In the present study, we demonstrated that hic-5 was one of the positive regulators in myogenic differentiation. This conclusion is in contrast with a previous report by Hu et al. that hic-5 blocked the differentiation of C2C12 cells (Hu et al., 1999). This apparent contradiction would be accounted for by the difference in strategy to elucidate the hic-5 function; they overexpressed hic-5 in the cells and observed that the hic-5-overexpressing clones were incapable of forming myotubes and instead died of apoptosis. We employed an antisense expression system to avoid the non-physiological response inevitable to ectopic overexpression of a protein, and rather reduced the expression level of hic-5. Recently, we have demonstrated the biological significance of hic-5 function in integrin signaling and characterized the mode of action of hic-5 as a competitor of paxillin, another adaptor protein.
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molecule involved in the integrin signaling (Turner, 2000; Nishiya et al., 2001). Thus, a small change in the amount of hic-5, either an increase or decrease, potentially causes a shift in the balance of the relative concentrations of hic-5 and paxillin, leading to quantitative and qualitative changes in integrin signaling. The profound effects of hic-5 expres-

Fig. 4. Myotube formation of clones with low levels of hic-5. (A) The G418-resistant colonies transfected with pEF-BOS-anti-hic-5 were isolated, and hic-5 protein expression levels were examined by Western blotting. The hic-5 level of individual clones was quantified using NIH image and normalized to that of the parental C2C12 population (designated as clone: C). (B) After expansion of individual clones, the medium was replaced with differentiation medium, and photographs were taken after 10 days. The value in parentheses is the hic-5 protein expression level of the clone, which was examined in (A).
sion on myogenic differentiation, evident from the antisense expression observed here and the overexpression in a previous report (Hu et al., 1999), could be the consequences of such an alteration of integrin signaling. Before reaching a conclusion, further experiments will be needed that elucidate the role of hic-5 at physiological conditions.

An intricate network controlling the process of morphogenesis and cell differentiation is composed of molecular machinery operating in signaling cascades, transcription complexes, cellular microenvironment and so on. It is thought that one of the functions of cell-ECM interactions or the cellular microenvironment in the above process is to maintain differentiated phenotypes of a variety of cells (Adams and Watt, 1993). Although ECM or its receptor, integrin, has been shown to be part of the above network, little is known about how it regulates the process. Additional study of the molecular basis of hic-5 functions will shed light on these issues. It would also provide a molecular tool to manipulate the fate of cells of mesenchymal origin such as fibroblasts, osteoblasts and myoblasts, whose phenotypes are modulated by hic-5 in common as demonstrated by others (Shibanuma et al., 1997; Shibanuma and Nose, 1998; Ishino et al., 2000; Hu et al., 1999) and the present study.

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References


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