ID – A dominant negative regulator of skeletal muscle differentiation – is not involved in maturation or differentiation of vascular smooth muscle cells

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Abstract Rat aortic vascular smooth muscle cells (VSMCs) expressing the MyoD gene formed myotubes at a low frequency in the presence of serum but at a high frequency in the absence of serum. Expression of an antisense Id gene increased myotube formation in the presence of serum indicating that a reduction in Id levels is a major mechanism by which serum withdrawal promotes myotube formation. The role of Id in the development of VSMCs was investigated by expressing an antisense Id gene in neonatal VSMCs. No evidence was found for the conversion of neonatal VSMCs to adult VSMCs in the presence of the antisense Id gene. Similarly reduction in Id by serum withdrawal also failed to cause conversion of the neonatal VSMCs to the adult phenotype. These data suggest that the maturation of neonatal smooth muscle cells is not controlled by a VSMC homologue of the skeletal muscle basic-helix-loop-helix proteins.

Key words: Maturation; Vascular smooth muscle cell; Id; MyoD; Differentiation

1. Introduction

The process of skeletal muscle myogenesis is relatively well understood [1]. Skeletal muscle myogenesis is controlled by at least 4 well-characterised proteins including MyoD [2], myogenin [3,4], Myf 5 [5] and MRF 4/herculin/Myf 6 [6]. These proteins are transcription factors of the basic helix-loop-helix (bHLH) family which act on the enhancer sequences of skeletal muscle specific genes [7]. MyoD, the first discovered and most studied of these proteins, binds to its recognition sequence either as a homodimer or as a heterodimer with the products of the E2A gene [8]. However, only the heterodimer efficiently activates transcription of muscle specific genes (e.g. muscle creatine kinase). The formation of dimers between MyoD and E2A gene products is antagonised by the protein Id [9], an HLH protein with no DNA binding domain. Id can dimerise with either MyoD or E2A gene products but since Id has a higher affinity for the products of the E2A gene it preferentially forms dimers with these proteins. Id/E2A heterodimers are unable to bind to DNA and hence the presence of Id prevents E2A binding to MyoD and activating muscle specific enhancers. Since Id is expressed at high levels in proliferating, undifferentiated myoblasts in the presence of serum and at low levels in non-proliferating, differentiated cells it is thought to inhibit differentiation whilst the cells are proliferating [9,10].

In contrast to skeletal muscle there is relatively little information on the process of smooth muscle myogenesis. We have previously investigated the relationship between Id expression and the differentiation of VSMCs marked by the levels of smooth muscle specific myosin heavy chain (SM-MHC) in rat aortic vascular smooth muscle cells (VSMCs) [11]. No correlation was found between Id expression and smooth muscle specific protein levels suggesting that Id does not regulate this differentiation process. However, this does not rule out a role for transcription factors regulated by Id at other points in the development of smooth muscle cells.

Cell culture studies have shown that VSMCs grown from neonatal rats and adult rats differ in their morphology and growth characteristics [12,13]. VSMCs cultured from aortae of neonatal rats have a cobblestone morphology and grow to monolayer confluence. These cells are also able to grow in platelet poor plasma suggesting that they produce an autocrine growth factor [13]. In contrast to neonatal rat VSMCs, cells cultured from the aortae of adult rats typically have a spindle morphology and grow to a 'hills and valleys' pattern at confluence. VSMCs derived from adult rats do not proliferate in platelet poor plasma suggesting that they do not produce any autocrine growth factor. As the animals age the VSMC phenotype appears to convert from the neonatal to the adult phenotype. We have developed in an in vitro model of the conversion of neonatal to adult VSMCs in which neonatal cells are treated with HMBA. To determine whether a smooth muscle-specific bHLH transcription factor could be regulating this process we have investigated the role of Id in this interconversion.

2. Materials and methods

2.1. Plasmids

To generate the sense and antisense Id constructs the Id gene was cut out of the vector pMH18ΔR [9] with *Eco*RI and *Bam*HI and the 0.9 kb fragment was purified. The overhanging ends of the purified fragment were filled in using the Klenow enzyme before the fragment was ligated into *Sma*I cut pMSG (Pharmacia). Vectors carrying Id gene inserts were identified by restriction digestion. The orientation of the inserts was determined by restriction mapping and confirmed by cloning *Xho*I/*Xba*I fragments of the pMSG-Id vectors into Bluescript KS$^+$ followed by double stranded sequencing using a Sequenase kit. The vector construct is shown in Fig. 1. All reactions were carried out as described in [14]. Plasmid DNA for transfections was prepared by PEG precipitation as described in [14].

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Abbreviations: VSMC, vascular smooth muscle cell; DMEM, Dulbecco's modification of Eagle's medium; FCS, foetal calf serum; BSA, bovine serum albumin; bHLH, basic helix-loop-helix; SK-MHC, skeletal muscle-myosin heavy chain; SM-MHC, smooth muscle myosin heavy chain; HMBA, hexamethylenebis-acetamide; MMTV, mouse mammory tumour virus.

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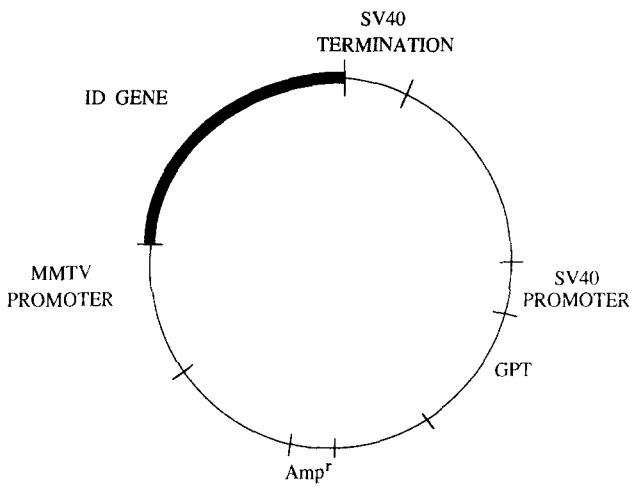

Fig. 1. Plasmid map for the pMSG Id constructs.

2.2. Cell culture and transfections

VSMCs from adult rat aortae, derived as described in [15] were maintained in DMEM supplemented with 10% FCS. Every 4 days cells were released by trypsin/EDTA, diluted 3-fold and plated in new flasks. A clone of neonatal VSMCs was derived from 12-day-old rats by a modification of the enzyme dispersal method previously described [15]. Briefly, cells dispersed from 12-day-old rat aortae were diluted to $4 \times 10^2 \cdot ml^{-1}$ in M199 + 10% FCS and plated into 96-well microplates at 40 cells $\cdot well^{-1}$. Arfter 28 days in culture, the cells in wells which contained a single colony were released with 25 μl of trypsin/EDTA at 37°C and replated into a fresh 96-well microplate containing 200 μl of M199 + 10% FCS. When the cells had reached confluence they were subcultured into two wells of a 96-well microplate by the same procedure. Cell populations were expanded by sub-culturing into progressively larger wells and finally maintained in 75 cm^2 flasks in DMEM + 10% FCS. Clones were karyotyped as described previously [16].

Both adult and neonatal VSMCs were infected as described in [17] with a MyoD cDNA containing retrovirus, MDSN, derived from LNL-6. Briefly, VSMCs were plated in 60 mm petri dishes at a density of $3 \times 10^4 \cdot cm^{-2}$. After 24 h the medium was replaced with DMEM + 4 $\mu l \cdot ml^{-1}$ polybrene and 100 μl viral stock (10^7 neomycin resistant cfu $\cdot ml^{-1}$). The medium was changed after 16 h to DMEM + 10% FCS supplemented with 50 $\mu g \cdot ml^{-1}$ geneticin. Cells were selected for 2 weeks prior to sense or antisense Id transfection and analysis for myotube formation.

Cells were transfected by electroporation as described in [18] using 40 μg of plasmid DNA. After electroporation the cells were allowed to recover for 10 min before 9.2 ml of DMEM + 10% FCS was added and the cells were seeded into 12-well plates at 0.5 ml $\cdot well^{-1}$. Using this procedure approximately 10% of the surviving VSMCs express β-galactosidase 24 h after transfection with the β-galactosidase expression vector pSVβgal (Promega).

24 h after electroporation, dexamethasone (1 μM) was added to initiate transcription of the sense and antisense Id genes. The cells were then either left for a further 72 h in the presence of 10% FCS and dexamethasone (1 μM), or the medium was changed after 24 h in the presence of serum and dexamethasone, to serum-free DMEM + dexamethasone (1 μM) and the cells were left serum free for an additional 48 h. The cells were then either fixed and stained for skeletal muscle myosin heavy chain (SK-MHC) or harvested and subcultured 1:10, 1:20 and 1:50 into DMEM + 10% FCS. After 2 days of culture at low cell density the cells were fixed and stained for SM-MHC. Control experiments were similar except that the dexamethasone was omitted.

2.3. Immunocytochemistry and Northern analysis

Cells for immunocytochemistry were washed three times in PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM) and fixed in 70% ethanol and 1% acetic acid for 2 min at room temperature. The cells were rehydrated in PBS and washed three times for 3 min each wash. SM-MHC was detected by immunoperoxidase staining carried out as described by [15] using a polyclonal antiserum raised against chicken gizzard myosin [19] an anti-rabbit FAB fragment raised in goat (ICN Flow) and an anti-goat IgG conjugated to peroxidase.

SK-MHC was stained using a mouse anti-chicken SK-MHC antibody MF20 [20]. After fixing and washing the cells were incubated in PBS + 3% BSA for 15 min then in MF20 diluted 1:5 in PBS + 3% BSA at 4°C for 2 h. The cells were washed three times with PBS for 3 min each wash and incubated with an anti-mouse IgG antibody conjugated to peroxidase for 1 h at room temperature prior to washing with PBS and DAB staining. Nuclei in cells staining positive for SK-MHC and containing at least 3 nuclei were counted as being myotube nuclei.

Fig. 2. Skeletal muscle myosin expression and myotube formation in MyoD expressing VSMCs. Cells were infected as described in section 2 and plated into 60 mm dishes in the presence of 10% FCS. After 72 h the cells were fixed and stained for skeletal muscle MHC.

Northern analysis was carried out as described in [11] using an Id cDNA probe. The Northern blot was autoradiographed on Fuji-RX X-ray film for 6 days at −70°C.

3. Results and discussion

3.1. Effect of Id on myotube formation in MyoD transfected VSMCs

Adult vascular smooth muscle cells were infected with a retrovirus containing the MyoD gene (MDSN). After selection in geneticin (50 $\mu g \cdot ml^{-1}$) for 2 weeks the cells (termed VSMC/MDSN) were passaged in the presence of 10% FCS and then incubated for 3 days before fixing and staining for SK-MHC. Under these conditions 4.5 ± 1.5% of the cells converted to myotubes (Figs. 2 and 3). When VSMC/MDSNs were plated in the presence of 10% FCS for 1 day then serum starved for 2 days prior to fixing and staining for SK-MHC, the proportion of cells forming myotubes had increased to 51.2 ± 5.6%. These experiments showed that under conditions where the amount of Id mRNA is reduced by serum starvation [11] there is an increase in the formation of myotubes. This is consistent with the work of [9] on the differentiation of myoblasts.

To test whether this effect was specifically due to lower Id mRNA levels or to serum withdrawal, further experiments were carried out in which the cells were transfected with a vector containing the Id gene in either the antisense (IdA) or sense (IdS) orientation under the control of the dexamethasone inducible MMTV promoter (as described in section 2). When cells transfected with IdA were maintained in 10% FCS for 3 days, 4.3 ± 1.1% of the cells formed myotubes. If dexamethasone (1 μM) was added to the cells 24 h after transfection the percentage of cells fusing into myotubes 2 days later increased to 9.7 ± 2.7%, $P < 0.05$, $n = 4$ (Fig. 4a). Under similar conditions addition of dexamethasone to IdS transfected VSMC/MDSNs reduced myotube formation (4.0 ± 1.1% to 1.2 ± 0.7%, $P < 0.05$, $n = 4$). A similar reduction in myotube formation was seen in cells electroporated in the absence of plasmid DNA (4.5 ± 1.5% to 1.1 ± 0.6%, $P < 0.05$, $n = 4$) (Fig. 4a). This means that the effective increase in myotube formation due to the IdA vector is 8.6% (from 1.1 ± 0.6% for cells electroporated in the absence of plasmid DNA in 10% FCS and 1 μM dexamethasone to 9.7 ± 2.7% ($P < 0.01$) for IdA transfected cells in 10% FCS and 1 μM dexamethasone). The transfection efficiency under these conditions has been found to be approximately 10% of the surviving cells (section 2). Therefore the increase in frequency of myotube formation suggests that most of the transfected cells expressing antisense Id convert into myotubes.

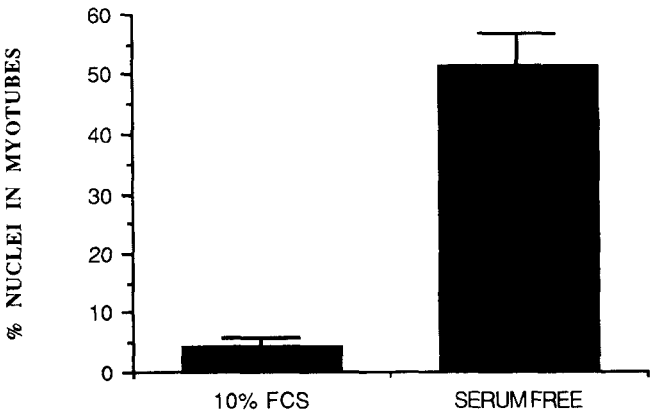

Fig. 3. Myotube formation of MyoD expressing VSMCs (VSMC/MDSNs) in the presence and absence of serum. Cells were plated into 12-well plates and grown for 72 h in the presence of 10% FCS, or plated into 12-well plates and grown for 24 h then serum starved for a further 48 h, before being fixed and stained for skeletal muscle myosin. A myotube was taken to be a cell staining positive for skeletal muscle MHC and incorporating at least 3 nuclei. The number of nuclei in myotubes is expressed as a percentage of total nuclei. Data are mean ± S.E.M. of $n = 4$ counts.

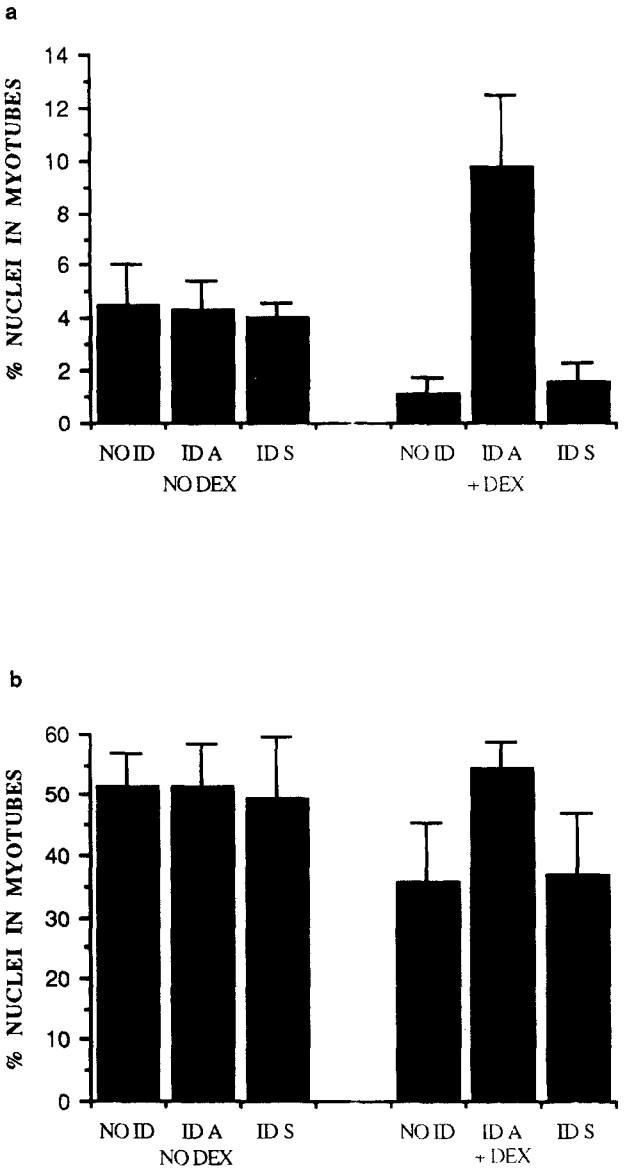

Fig. 4. Myotube formation of VSMC/MDSNs in response to antisense Id expression. Cells were electroporated with in the absence of plasmid DNA (NO ID), in the presence of 40 μg of IdA or the presence of 40 μg IdS. (a) After 24 h in the presence of 10% FCS dexamethasone (1 μM) was added and the cells were fixed 72 h later. (b) After 24 h in the presence of 10% FCS dexamethasone (1 μM) was added. 24 h later the cells were put into serum-free DMEM for 48 h before they were fixed and stained for skeletal muscle MHC. Data are expressed as percentage of total nuclei in myotubes derived as in Fig. 3 (mean ± S.E.M. of $n = 4$ counts).

Serum withdrawal increased myotube formation in VSMC/MDSNs transfected with the IdS vector and in cells electropo-

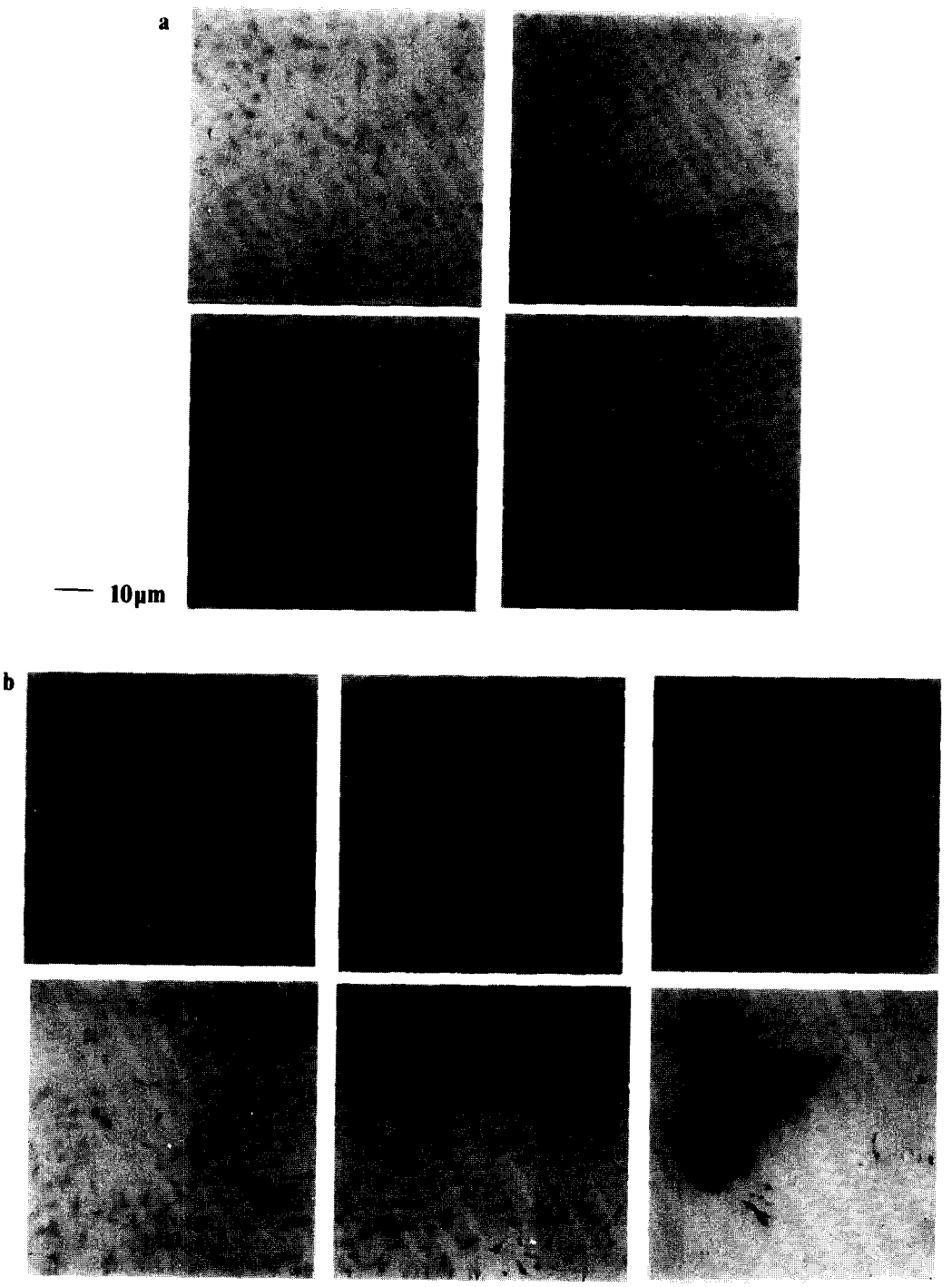

Fig. 5. Interconversion of VSMCs. (a) Confluent neonatal cells (upper panels) and adult cells (lower panels) were released with trypsin/EDTA, resuspended in DMEM + 10% FCS and plated into 96-well plates at normal (1:3, left hand panels) and low (1:20, right hand panels) cell density. The cells were grown for 48 h in DMEM + 10% FCS, fixed and stained for SM-MHC content (as described in section 2). (b) Neonatal cells were subcultured (1:3) in DMEM + 10% FCS and grown in 96-well plates. Cells in one well were then fixed and stained for SM-MHC (bottom left panel). Cells in another well were photographed under phase contrast (top left panel). The medium in the remaining wells was changed to DMEM + 10% FCS + 10 mM HMBA and the cells were incubated for a further 48 h. Cells in one well were then fixed and stained for SM-MHC (bottom middle panel). Cells in another well were photographed under phase contrast (top middle panel). The medium in the remaining wells was changed to DMEM + 10% FCS and the cells were grown to confluence. The resultant cells were subcultured and diluted 1:20 in DMEM + 10% FCS and grown for 48 h. Cells in one well were then fixed and stained for SM-MHC (bottom right panel). Cells in another well were photographed under phase contrast (top right panel). The micrographs show that neonatal cells can be converted into an adult cell phenotype and both adult and treated neonatal cells convert to a large cell phenotype when plated at low cell density.

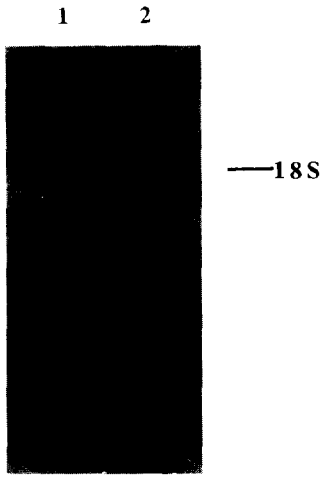

Fig. 6. Serum withdrawal reduces Id expression in neonatal cells. RNA extracted from neonatal/MDSN cells subcultured 1:10 in DMEM + 10% FCS incubated for 24 h then put into serum-free medium for 3 days and washed every day with serum-free DMEM. Lane 1 = RNA extracted from type C/MDSNs subcultured 1:10 and grown in DMEM + 10% FCS for 4 days; lane 2 = RNA was hybridised to an Id cDNA probe, washed as described in Kemp et al. (1991) and exposed to X-ray film at −70°C.

rated in the absence of plasmid DNA from 4.0 ± 0.6% to 49.5 ± 10.1% and 4.5 ± 1.5% to 51.2 ± 5.6% respectively (Fig. 4). Under serum-free conditions, dexamethasone (1 μM) reduced myotube formation of both IdS transfected cells and cells electroporated in the absence of plasmid DNA (Fig. 4b). Serum withdrawal also increased myotube formation of VSMC/MDSNs transfected with the IdA vector (to 51.5 ± 6.9%). However, the addition of dexamethasone under these conditions had no effect on myotube formation in these cells (Fig. 4b).

Taken together these data suggest that the increased formation of myotubes by the VSMC/MDSNs upon serum withdrawal is due to the reduction in Id expression. The data are in agreement with the findings of [9] who showed that Id prevented MyoD-dependent expression from the muscle creatine kinase promoter and the experiments of [10] which showed that ectopic expression of Id inhibited the skeletal muscle differentiation program of C2C12 myoblasts. The data are also consistent with the hypothesis that Id is a negative regulator of MyoD transactivation of myogenesis and indicate that Id is capable of inhibiting the expression of genes dependent on MyoD and E2A in VSMCs. Consequently the data suggest that Id could act as an inhibitor of putative bHLH transcription factors involved in smooth muscle myogenesis.

3.2. Maturation of VSMCs

We, therefore, investigated whether Id regulates the maturation step of smooth muscle myogenesis using the IdA and IdS expression vectors. As a model of VSMC maturation, neonatal cells were incubated for 24 h in the presence of HMBA (10 mM) then washed and allowed to grow on to confluence. The normal cobblestone morphology, typical of neonatal cells, was converted to the spindle morphology of adult cells (Fig. 5). One property of adult but not neonatal VSMCs is that when plated at low cell density (1:20) adult but not neonatal VSMCs convert into a large non-proliferative phenotype which contains large amounts of SM-MHC (Fig. 5). The converted cells plated at this low density also changed into a large non-proliferative phenotype which stained strongly for SM-MHC protein (Fig. 5). Taken together these observations indicate that the cells had matured into the adult VSMC phenotype. The formation of large non-proliferative VSMCs which stain strongly for SM-MHC can also be used as a single cell assay for maturation. Both HMBA and similar compounds have been shown to induce differentiation of other cell types (e.g. erythroleukemia cells). Additionally in erythroleukemic cells a reduction in Id gene expression has been shown to occur coincidentally with differentiation induced by such agents [9].

3.3. The effect of Id on VSMC maturation

To test the hypothesis that a reduction in Id expression promotes maturation neonatal cells were transfected with the IdA or IdS vectors. The cells were maintained in DMEM + 10% FCS for 24 h after transfection then dexamethasone (1 μM) was added and the cells were grown for 3 days before being subcultured to low density (1:10, 1:20 and 1:50) incubated for 2 days in DMEM + 10% FCS then fixed and stained for SM-MHC. Amounts of SM-MHC protein did not increase in any of the cells in the presence of the IdA or IdS vectors. Cells electroporated in the absence of plasmid DNA also failed to increase their SM-MHC content. None of the cells underwent the morphological change to the large smooth muscle cell phenotype. This indicates that antisense Id expression did not cause the maturation of the neonatal VSMCs to adult VSMCs. One possible explanation for these observations would be very high Id expression in the neonatal cells. To reduce the level of Id mRNA in the neonatal cells they were withdrawn form the cell cycle by a process of repeated washing with serum free medium and then examined for conversion to adult cells. Neonatal cells were plated at low density (1:10) and 24 h later the cells were washed three times with serum-free medium and then incubated in serum free medium. Every 24 h for 3 days these cells were washed three times with serum free medium then incubated in serum free medium. On the fourth day in serum free medium the cells were either harvested by trypsinisation for Northern analysis of their Id mRNA content or subcultured at low density (1:10, 1:20 or 1:50) into DMEM + 10% FCS. After a further 48 h in culture the cells were fixed and stained for SM-MHC. Id mRNA levels were significantly reduced by this protocol (Fig. 6) however, the neonatal cells did not increase their SM-MHC content or change morphology to the large VSMC phenotype indicating that they did not convert into adult cells.

These data suggest that Id is not involved in the conversion of neonatal VSMCs to adult type VSMCs and that the conversion of neonatal to adult VSMCs by HMBA is not regulated by Id. The data also agree with a previous study which showed that transcription factors which regulate SM-MHC gene expression [11] are not regulated by Id since an increase in SM-MHC gene expression was not observed in VSMCs expressing antisense Id. In conclusion the data demonstrate that Id can function as a dominant negative regulator of MyoD in the presence of serum in an MyoD transfected adult VSMCs. However, the process of VSMC maturation was not regulated by the interaction of a smooth muscle specific bHLH transcription factor with a product of the E2A gene or any other bHLH factor which interacts with Id.

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