Accumulation of 210 kDa microtubule-interacting protein in differentiating P19 embryonal carcinoma cells

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Abstract The MA-01 antigen, a thermolabile 210 kDa microtubule-interacting protein, is present in P19 embryonal carcinoma cells on microtubular structures as well as in cytosol. After aggregation of the cells and subsequent incubation with all-*trans*-retinoic acid (RA), the level of MA-01 expression increased approximately 10 times during 15 days. The increase started after 2 days of incubation with RA and preceded the appearance of neuron-specific tubulin βIII, MAP2C and neurofilaments. Such elevated expression of MA-01 antigen was not detected in P19 cells treated with dimethylsulfoxide. These data indicate that enhanced expression of MA-01 antigen is one of the earliest events occurring in P19 cells during neuronal differentiation.

Key words: Antibody; Microtubule-interacting protein; P19 cell; Retinoic acid

1. Introduction

Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, provide a suitable model system for studying the molecular mechanisms underlying differentiation and early embryonic development. P19 are murine multipotent EC cells that can differentiate in culture into neurons, glial cells and fibroblast-like cells when aggregated and cultured in the presence of a non-toxic concentration of all-*trans*-retinoic acid (RA) [1]. On the other hand, if the P19 cells are treated with 1% dimethylsulfoxide (DMSO), they form smooth, cardiac and striated muscle cells [2]. P19 cells have been used as a differentiating model system that makes it possible to examine the role of tubulin isoforms and microtubule-associated proteins in early stages of the development of neuronal cytoskeleton [3–5]. In addition to cytoskeletal genes that are predominantly expressed in P19 cells committed to differentiate along the neuronal pathway, RA also induces the expression of a whole range of other genes implicated in the control of early development [6].

Here, we report on the distribution and expression of MA-01 antigen, a thermolabile 210 kDa microtubule-interacting protein [7] in P19 cells stimulated to neuronal differentiation. The data document that an elevated expression of MA-01 antigen is one of the earliest events observed in P19 cells differentiating into neuronal cells.

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Abbreviations: DMSO, dimethylsulfoxide; EC, embryonal carcinoma; RA, all-*trans*-retinoic acid

2. Materials and methods

2.1. Cells

P19.X1 cells, a subclone of mouse EC cells P19 [8], were cultured in basic culture medium that was prepared by mixing the Dulbecco's modified Eagle's medium and RPMI 1640 medium (1:l, v/v) containing 10% (v/v) fetal bovine serum, penicillin (l00 U/ml) and streptomycin (0.1 mg/ml). Cells were grown at 37°C in 5% CO_2 in air and passaged every 2 days using 0.02% EDTA in 0.15 M NaCl/0.01 M phosphate, pH 7.5. Cell aggregates were formed by plating 10^5 cells per ml into bacteriological-grade Petri dishes. After 2 days, the cell aggregates were gently spun down, resuspended in medium containing 10^{-6} M RA (Sigma-Aldrich, Prague, Czech Republic) and transferred into tissue culture dishes (diameter 6 cm) in aliquots of 10^5 per dish. Medium containing RA was changed every 2 days. For immunofluorescence studies, the cells were cultured on glass coverslips.

2.2. Antibodies

The following monoclonal antibodies were used. The MA-01 antibody (IgG1), raised against porcine brain microtubule-associated protein MAP2, reacts with a 210 kDa protein in different non-neuronal cells of various species and decorates microtubule structures [9,10]. The antibody MA-02 (IgG1) is directed against a different epitope of the same protein (Dráberová, unpublished). α-Tubulin was detected by the TU-01 antibody (IgG1) [11], neuron-specific class III β-tubulin by the TUJ1 antibody (IgG2a) (BAbCO, Richmond, CA, USA), MAP2C by the HM-2 antibody (IgG1) (Sigma-Aldrich, Prague, Czech Republic), neurofilament triplet protein NF-M by the antibody NF-09 (IgG2a) [12] and vimentin by the antibody VI-10 (IgM) [13]. Actin was detected by rabbit affinity-purified antibody against the C-terminal peptide (Sigma-Aldrich, Prague, Czech Republic), and MAP2AB by rabbit affinity-purified antibody (generous gift from Dr. V. Gelfand, Moscow State University). In double-label immunofluorescence, microtubular structures were detected by a rabbit affinity-purified antibody against αβ-tubulin dimer [14]. The monoclonal antibody HTF-14 (IgG1) against human transferrin [15] was used as a negative control. The purified antibody MA-01 was conjugated with biotin using sulfosuccinimidyl-6-(biotinamido)hexanoate (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL, USA) according to the manufacturer's direction with the antibody/biotin weight ratio 160/1. Anti-mouse Ig antibodies and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI, USA), lissamine rhodamine-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate-conjugated anti-rabbit Ig antibody were from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

2.3. Preparation of cell extracts and immunoprecipitation

For the preparation of whole cell extracts, cells on 60 mm Petri dishes were rapidly rinsed twice in MEM buffer (0.1 M Mes adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$), solubilized in hot sodium dodecyl sulfate (SDS) sample buffer [16] without bromophenol blue (0.7 ml/dish) and boiled for 5 min. For preparation of soluble and cytoskeletal fractions at 37°C, cells on Petri dishes were rapidly rinsed twice in MEM buffer and then extracted with 0.6 ml of MEM buffer supplemented with protease inhibitors (1 μg/ml each of leupeptin, aprotinin, antipain, pepstatin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) and containing 0.2% (v/v) Triton X-100. After 2 min, the extract was gently removed and one-fourth volume of 4×SDS sample buffer was added. The cytoskeletons remaining on the plate were gently rinsed once with MEM buffer containing inhib-

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itors and solubilized with 0.7 ml of SDS sample buffer. Samples were boiled for 5 min.

For immunoprecipitation, cells were rinsed in cold MEM buffer supplemented with inhibitors and harvested by means of a rubber policeman into the same buffer (0.6 ml/dish). Cells were then disrupted on ice by sonication for 3×20 s (amplitude 40) with a 500 W Ultrasonic homogenizer, (Cole-Parmer, Vernon Hills, IL, USA) equipped with a microtip probe. The resulting homogenate was centrifuged at 40 000×*g* for 30 min (4°C). The supernatant was then used for immunoprecipitation. Anti-mouse antibody was covalently coupled to CNBr-activated Sepharose 4B and the beads were equilibrated in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). 80 μl of sedimented beads with the immobilized anti-mouse antibody was incubated under rocking at 4°C for 2 h with 1 ml of MA-01 antibody at a concentration of 0.5 mg/ml in TBST or with HTF-14 antibody at the same concentration. The beads were pelleted by centrifugation at 4000×*g* for 30 s, washed four times (5 min each) in cold TBST, and further incubated under rocking for 3 h at 4°C with 0.5 ml of cell extract diluted 1:1 with TBST. The beads were pelleted and washed four times, for 5 min each, in cold TBST before boiling for 5 min in 100 μl of SDS sample buffer to release the bound proteins.

2.4. Gel electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [16]. The boiled samples were centrifuged at 22 000×*g* for 10 min and the protein concentration in supernatants was determined by the silver-staining method as described [17] using BSA (Serva Feinbiochemica, Heidelberg, Germany) as a standard. Proteins were electrophoretically transferred from gels onto nitrocellulose sheets according to Towbin et al. [18]. Details of the immunostaining procedure are described elsewhere [19]. The MA-01 antibody was used at a concentration of 1 μg/ml. The antibodies TU-01 and NF-09, in the form of ascitic fluids, and antibody TUJ1 were diluted 1:2000, the antibody HM-2 was diluted 1:500. The anti-actin antibody and anti-MAP2AB antibody were diluted 1:100. The antibody VI-10, in the form of spent culture supernatant, was diluted 1:10. Bound antibodies were detected by incubation of blots with secondary antibodies, diluted 1:10 000, and after washing with enhanced chemiluminescence (ECL) reagents according to the manufacturer's directions (Amersham, Little Chalfont, UK). ECL reagent was also used for detection of bound biotinylated MA-01 antibody after incubation of blots with ExtrAvidine peroxidase conjugate (Sigma-Aldrich, Prague, Czech Republic), diluted 1:5000. Autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY, USA) were quantified by densitometry using gel documentation system GDS 7500 and GelBase/GelBlot Pro analysis software (UVP, Upland, CA, USA).

2.5. Immunofluorescence

Immunofluorescence microscopy was performed on fixed cells as described [7]. Shortly, cells were rinsed briefly with MSB (MEM buffer supplemented with 4% polyethylene glycol 6000), extracted for 1 min with 0.2% Triton X-100 and fixed for 20 min in 3% formaldehyde in MSB. Preparations were washed three times in MSB and incubated for 45 min at room temperature with MA-01 antibody at a concentration of 5 μg/ml. For double-label immunofluorescence staining, the coverslips were incubated simultaneously with MA-01 and polyclonal anti-tubulin antibody diluted 1:5, and after washing incubated simultaneously with secondary fluorochrome-conjugated antibodies diluted 1:100. The preparations were mounted in MOWIOL 4-88 (Calbiochem AG, Lucerne, Switzerland) and examined with an Olympus A70 Provis microscope equipped with 60× and 20× water-immersion objectives and DIC. Images were recorded with a Life Science Resources KAF 1400 cooled CCD camera. Neither the control antibody HTF-14 nor the conjugates alone gave any detectable staining.

3. Results and discussion

Double-label immunofluorescence microscopy with polyclonal anti-tubulin antibody showed that the MA-01 antibody decorated interphase and mitotic microtubule arrays in resting P19 cells (Fig. 1A,D). The antibody also decorated tubulin paracrystals and bundled microtubules in cells treated, respec-

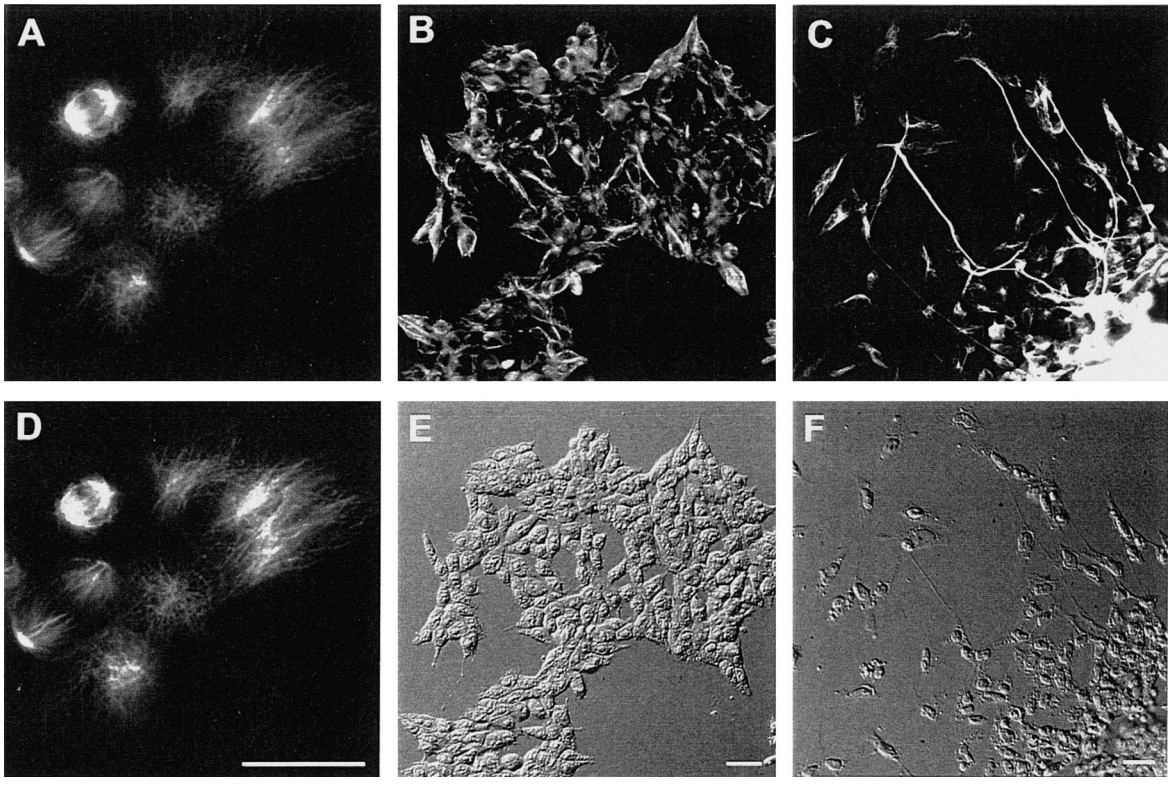

Fig. 1. Immunofluorescence staining of resting and activated P19 cells with MA-01 antibody. Staining of unstimulated cells (A, B) and cells stimulated by RA for 6 days (C) with the MA-01 antibody (A–C). Double-label staining with polyclonal anti-tubulin antibody (D) is shown in A–D. Differential contrast microscopy is shown in E and F. Each pair (A–D, B–E and C–F) represents the same cells. Bar, 20 μm.

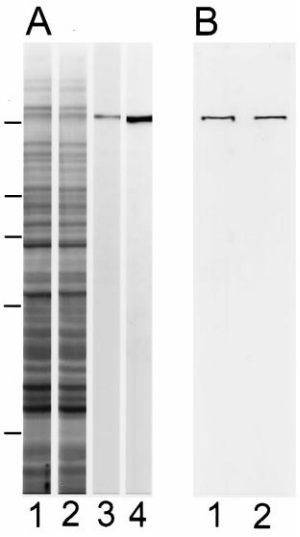

Fig. 2. Immunoblot of resting and activated P19 cells with MA-01 antibody. A: Unstimulated cells (lanes 1 and 3) and cells stimulated by RA for 6 days (lanes 2 and 4). Lanes 1 and 2, Coomassie blue staining of separated proteins; lanes 3 and 4, immunostaining. B: Soluble (lane 1) and insoluble (lane 2) fraction of unstimulated cells treated for 2 min at 37°C in 0.2% Triton X-100. 7.5% SDS–PAGE. 10 μg of protein was loaded per lane. Bars on the left margin indicate position, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 66 and 45).

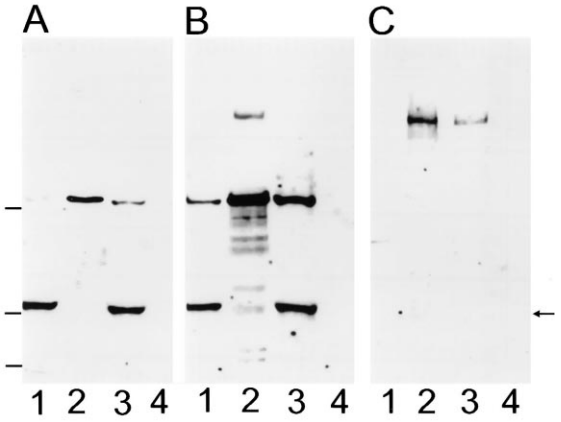

Fig. 3. Immunoblot of P19 cells immunoprecipitated by MA-01 antibody. A: Unstimulated cells. B and C: Cells stimulated by RA for 9 days. Cell extracts were precipitated with MA-01 antibody (lanes 1 and 2) or with control HTF-14 antibody (lanes 3 and 4). Proteins remaining after precipitation (lanes 1 and 3) and immunoprecipitated proteins (lanes 2 and 4) were separated on 5% SDS–PAGE. A and B: Immunostaining of blotted proteins with biotinylated MA-01 antibody. C: Immunostaining of blotted proteins with anti-MAP2 antibody. Bars on the left margin indicate position, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 66 and 45). Arrow indicates reaction of extravidin in cell extracts.

tively, with vinblastine or taxol (not shown). In nocodazole-treated cells, the antibody gave diffuse staining and no microtubules were detected (not shown). After aggregation of the cells and their incubation in the presence of 10^{-6} M RA, long neuritic projections were detected. A comparison of resting and activated cells (6 days with RA) by differential contrast microscopy is shown in Fig. 1E,F. Identical samples stained with MA-01 antibody are shown in Fig. 1B,C. The antibody decorated all cells and stained the long projections. We have observed that cells were more intensely stained already after 3 days of incubation with RA, although no long projections were recognizable.

Immunoblot analysis of whole extract of cells incubated with RA for 6 days revealed that the MA-01 antibody reacted with 210 kDa protein and that the amount of the 210 kDa antigen was higher in stimulated than in resting cells (Fig. 2A). Extraction of unstimulated P19 cells in 0.2% Triton X-100 at 37°C showed that MA-01 antigen was present in insoluble (cytoskeletal) as well as in soluble fractions (Fig. 2B). Immunoprecipitation experiments showed that 210 kDa antigen could be specifically precipitated from unstimulated (Fig. 3A, lane 2) as well as from stimulated cells (Fig. 3B, lane 2). In the latter case, the amount of the 210 kDa protein was substantially higher. From stimulated cells, the MA-01 antibody precipitated also 280 kDa protein that was subsequently detected by the MA-01 antibody. This band was not visible in experiments using cell extracts, and was only observed in concentrated precipitated material when a sensitive detection technique with biotinylated MA-01 antibody was employed. As it is known that the MA-01 reacts with MAP2 proteins [9,20], immunostaining of precipitated material from stimulated cells with polyclonal affinity-purified anti-MAP2 antibody was performed. Polyclonal antibody did stain the 280 kDa protein but not the 210 kDa band (Fig. 3C, lane 2). No staining with anti-MAP2 antibody was detectable with precipitated material from unstimulated cells. We also tested a panel of our and commercial monoclonal antibodies against MAP2, and none of them reacted with the 210 kDa band in either unstimulated or stimulated cells. Moreover, the 210 kDa protein is thermolabile in both unstimulated and stimulated cells (not shown), in contrast to the high molecular weight MAP2AB proteins. The 210 kDa protein in stimulated cells therefore did not represent a fragment of MAP2AB. Control antibody of the same class (HTF-14) precipitated the 210 kDa protein neither in unstimulated (Fig. 3A, lane 4) nor in stimulated cells (Fig. 3B, lane 4). Nor did the control antibody precipitate the MAP2 proteins (Fig. 3C, lane 4).

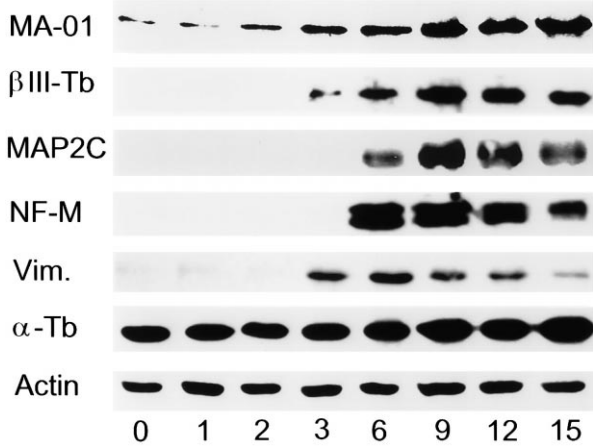

Fig. 4. Immunoblot analysis of whole cell extracts from unstimulated cells (day 0) and cells stimulated with RA for 1–15 days. The extracts (10 μg/lane) were separated on 5% (MA-01, NF-M) or 7.5% SDS–PAGE (βIII-Tb, MAP2C, Vim., α-Tb, actin). The βIII-Tb denotes neuron-specific class III β-tubulin, the Vim. denotes vimentin and the α-Tb denotes α-tubulin. Numbers 0–15 indicate days of incubation of cells with RA.

When the blotted proteins were probed only with horseradish peroxidase-labeled extravidin, a band in the position around 118 kDa was detected in cell extracts (arrow in Fig. 3). Consequently, it does not represent any specific staining with MA-01 antibody.

In order to investigate the relationship between the expression of MA-01 antigen and that of other cytoskeletal proteins during neuronal differentiation of P19 cells, blots of whole cell extracts from unstimulated cells and cells stimulated with RA for 1–15 days were probed with MA-01 antibody and with antibodies against cytoskeletal proteins. Antibodies against neuron-specific class III β-tubulin, MAP2C and against neurofilament triplet protein NF-M were used as markers of neuronal differentiation, antibodies against α-tubulin and against actin were used as controls. Expression of vimentin was also investigated. Typical results are shown in Fig. 4. Day 0 represents cells that were aggregated but not incubated in the presence of RA. The same amount of proteins was loaded at each time interval. An increased expression of the MA-01 antigen appeared after 48 h incubation of cells with RA and reached its maximum after 12 days. We have obtained the same results with MA-01 antibody and with MA-02 antibody. The class III β-tubulin was not detected in unstimulated cells and began to appear after 3 days of incubation with RA. The amount of class III β-tubulin started to decrease on day 15. MAP2C was not immunodetected with HM-2 antibody in unstimulated cells but it was clearly detectable in cells treated 6 days with RA. Its amount started to decrease on day 12. The NF-M protein was detected neither in unstimulated cells nor in cells treated for 3 days with RA. On the sixth day of stimulation, the expression of NF-M was found highly enhanced. The NF-09 antibody reacted with two proteins, which may possibly reflect the fact that the antibody reacts with both the phosphorylated and unphosphorylated form of NF-M [12]. Alternatively, this protein could have been proteolytically degraded during the sample preparation. The amount of detected NF-M and class III β-tubulin started to decrease on day 12. A very low level of vimentin was immunodetected on blots with VI-10 antibody already in unstimulated cells and in cells stimulated with RA for 1 day or 2 days. A substantial increase in vimentin expression was, however, detected on day 3. The amount of vimentin started to decrease on day 9. During the differentiation, the amount of detected α-tubulin rose only moderately. The used anti-tubulin antibody TU-01 reacts with an epitope located in the conservative N-terminal region and thus recognizes all post-translationally modified α-tubulins [11]. The amount of detected actin did not change during differentiation.

Densitometric measurements of immunoblots clearly showed that the increase in the expression of MA-01 antigen preceded the appearance of class III β-tubulin (Fig. 5) as well as vimentin (not shown). In the time interval between day 0 and day 15, the amount of MA-01 antigen increased approximately 10 times and this high level was still maintained after 18 days of incubation of cells with RA (not shown). In the same time interval, the amount of α-tubulin increased 1.5 times. The amount of detected MA-01 antigen was similar in resting (non-aggregated) and in aggregated cells (day 0). Aggregation itself therefore did not trigger the expression of this protein. The observed increase in the expression of MA-01 antigen after RA treatment seems to be specific for differentiating P19 cells. It is known that RA in some cell lines causes transcriptional induction of various proteins. For example the human promonocytic cell line U937 can be induced to terminal monocytic differentiation by RA [21]. We have observed that in stimulated U937 cells, the number of macrophage-like cells was substantially increased, as determined by the staining of nuclei with DNA-binding dye, after 5 days culturing of the cells in the presence of 10^{-6} M RA. However, no increase in MA-01 antigen expression was detected by immunofluorescence or by immunoblotting. Our data indicate that the elevated expression of MA-01 antigen in P19 cells is associated with the differentiation along neuronal pathway. If aggregated P19 cells are treated for 6 days with 1% DMSO known to stimulate muscle differentiation [22], no increase in MA-01 antigen expression was observed (not shown).

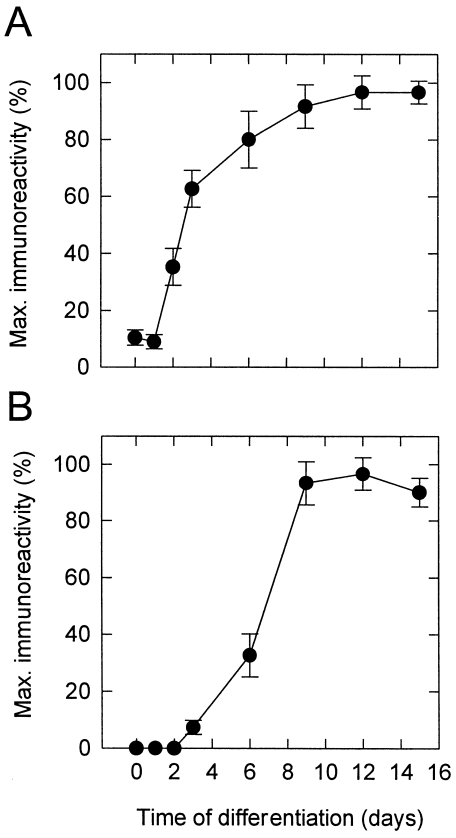

Fig. 5. Comparison of expression of MA-01 antigen (A) and class III β-tubulin (B) in unstimulated cells (day 0) and cells stimulated with RA for 1–15 days as detected by densitometric measurements of immunoblots. Each point on the graph represents the mean ± S.E.M. of duplicate readings of three blots.

It has been reported that unphosphorylated neuron-specific class III β-tubulin is the earliest neuronal lineage marker known so far [23]. Similarly, the class III β-tubulin, detected by TUJ1 antibody, appeared in very early stages of neuronal differentiation in non-aggregated P19 cells stimulated by RA [24]. We have detected class III β-tubulin in aggregated and RA-treated P19 cells on day 3, when the expression of MA-01 antigen was already increased four times. Chronological expression of MAP2AB, MAP2C, MAP1A and phosphorylated MAP1B was studied in P19 cells [4]. In that case, cells were induced to neuronal differentiation by absorbing cell aggregation in 3×10^{-7} RA for 4 days, induced aggregates were then dissociated and the cells incubated in serum-free medium.

Under such conditions, MAP2C and phosphorylated MAP1B appeared 12 h after the neuronal induction. Correlation with the expression of class III β-tubulin was not studied in that system [4]. Our data indicate that increased expression of MA-01 antigen preceded expression of MAP2C.

Previously, we have shown that the MA-01 antigen could be directly or indirectly involved in the interaction of microtubules and vimentin filaments [7]. There exist data showing that the microtubule motor protein kinesin participates in such interactions [25,26]. Upregulation of the vimentin gene in P19 cells aggregated for 3 days in the presence of RA was described [6], Here, we show that an increased expression of MA-01 antigen precedes the increased expression of vimentin. However, while the amount of vimentin decreased during neuronal differentiation, the amount of MA-01 antigen was kept at an increased level. It is possible that the changes in the expression of MA-01 antigen preceeding the expression of vimentin filaments and neurofilaments could somehow reflect its involvement in interactions between microtubules and vimentin filaments or neurofilaments in the course of neuronal differentiation of P19 cells. In conclusion, the presented data indicate that an increased expression of MA-01 antigen represents one of the earliest events during neuronal differentiation of P19 cells.

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