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Results: 2

1. A teratoproteomics analysis: heat shock protein 70 is upregulated in mouse forelimb bud by methoxyacetic acid treatment.
   Royani A, Sudarwati S, Sulasturya I.A, Sumarsono SH, Kim DJ, Chung JH.
   PMID: 32699587 [Published - indexed for MEDLINE]

2. The lamrin binding protein p40 is involved in inducing limb abnormality of mouse fetuses as the affects of methoxyacetic acid treatment.
   Royani A, Sudarwati S, Sulasturya I.A, Sumarsono SH, Giao T.
   PMID: 12605644 [Published - indexed for MEDLINE] Free Article
The laminin binding protein p40 is involved in inducing limb abnormality of mouse fetuses as the effects of methoxyacetic acid treatment.


Abstract

This study is intended to characterize a protein that is linked with mouse limb teratogenicity as the effects of methoxyacetic acid (MMA) treatment. A single dose of MAA (10 mmol/kg body weight) was given by gavage on gestation day (GD) 11, whereas the control group were administered vehicle only. The pregnant mice were killed at 4 h after MAA treatment, and forelimb buds were isolated from both the control and treated group embryos. Proteins from forelimb buds GD 11 + 4 h, which were precipitated out using 40-60% ammonium sulfate, then were analyzed by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2-D SDS-PAGE) technique. The 2-D gels reveal one protein with 41.8 kDa and pI 6.4, which expression was downregulated after MAA treatment. Tentative protein identification via peptide mass database search and definitive protein identification via a primary sequence database search indicate that the protein matches exactly to 34/57 kDa laminin binding protein (LBP; P14206, SwissProt), which is encoded by p40 gene (MGI: 1553581). The identity was further verified by Western blotting with an antibody against the 67 kDa LBP. The results suggest that MAA treatment to pregnant mice downregulates the LBP-p40 in the forelimb buds.
The Laminin Binding Protein p40 Is Involved in Inducing Limb Abnormality of Mouse Fetuses as the Effects of Methoxyacetic Acid Treatment

Acavi Bayduri*, Sri Sidiawati*, Lien A. Sutisayu*, Sony H. Sumarsono* and Forsten Gloe*

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Received January 21, 2003.
Accepted May 23, 2003.

Abstract
This study is intended to characterize a protein that is linked with mouse limb teratogenicity as the effects of methoxyacetic acid (MAA) treatment. A single dose of MAA (10 mM/mL) by body weight was given by gavage on gestation day GD 11, whereas the control group was given propylene glycol alone on GD 11 (Hoffman et al., 1993). The results indicate that cell death is induced in the mesenchyme tissue and, to some extent, in the epitheloid ridge (MER) after administration of 100 mg/mL MAA to the dams on GD 11. The teratogenic effects of MAA have been associated with the primary metabolite, methoxyacetic acid (MAA), which is catalyzed by aldehyde dehydrogenase and aldehyde dehydrogenase in the rat dam's liver. MAA and MAA have similar biological activities in mice, therefore their dose-response characteristics for producing developmental toxicity are nearly indistinguishable (Davies et al., 1994).

Several investigators reported that, after a single dose of MAA (10 mM/mL) by body weight, more than 50% of fetal limbs showed abnormalities, such as extrascapulae, brachydactyly, and syndactyly. When the substance was administered on GD 11 in ICR (Institute of Cancer Research, 1993) and Swiss Webster (Ward et al., 1994) mice, the fetal abnormalities were caused mainly by decreased cell death in the mesoderm of the limb plate (Hoffman et al., 1993; Davie et al., 1993). The AER also plays a role in inducing limb abnormalities, because it expresses rapidly in MAA-treated limbs compared to the control (Davie et al., 1993). It is well established that AER function is crucial for stimulating the proliferating zone of mesenchymal cells. A single dose of MAA (10 mM/mL) by body weight on GD 11 in NIH mice caused a decrease of weight, total protein, and RNA content at GD 11 + 6 h, +12 h, +24 h, and +48 h when compared with controls (Davies et al., 1994). Following a similar experimental design, we could show earlier that, in crude proteins from fetal limbs of GD 11 + 6 h, +12 h, +24 h, and +48 h, and +12 h, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1-D SDS-PAGE) protein bands within the range of 29-45 kDa were already decreased after 4 h of MAA administration compared to the controls (Bayduri et al., 2001). We therefore conducted this study to further investigate the identity of such regulated proteins.

MATERIALS AND METHODS

Experimental animals.
The mice were used as experimental animals. Twelve mice were divided into two groups of 23-27°C and 28°C humidity. Food and water were given ad libitum. When female mice achieved their sexual maturity (10-12 weeks old), they were mated with a male (1:3). A vaginal plug detected the following morning was defined as day 0 of pregnancy (Davie et al., 1995).

Materials, dosage, and sample collection.
Mice were sacrificed by cervical dislocation 4 h after MAA treatment (Bayduri et al., 2003). Fetal limb buds were isolated and stored at -85°C until final analysis.

Proteins precipitation.
Fetal limb bud samples were homogenized at 4°C in 3 volumes 40 mM sodium phosphate, pH 7. Cellular debris was separated by centrifugation at 4,350 x g, 4°C, for 20 min (Beckman J-20/60). The final obtained supernatants were isolated as crude-extract proteins.

Proteins of one part of the crude-extracts were precipitated for 30 min using 40-60% ammonium sulfate at 4°C. Precipitates were collected by centrifugation at 17,400 x g, 4°C, for 20 min (Beckman J-20/60, Refriger and Edison 1993). The protein fraction was solubilized completely with 40 mM sodium phosphate, pH 7, dialyzed on cellulose membranes (Nenh, 1985), and stored at 2°C as samples for two-dimensional electrophoresis.

Two-dimensional electrophoresis.
The two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2-D SDS-PAGE) technique was applied according to Debarbouil et al. (1990). The separation of proteins in the first dimension was performed according to their pl using two kinds of ampholytes (3.5-10 and 5-9) followed by separation of the second dimension according to their molecular weight using the SDS-PAGE (Coomassie, 1990). The 2-D electrophoretograms were performed in mini format. Control and treated protein samples, each of 75 pg (Bio-Rad and Kronke, 1990; Seto et al., 1995), were electrophoresed in accordance with the instruction manual (Bio-Rad).
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Before running the second dimension, the tube gels were equilibrated in SDS buffer for 15 min. The tube gels were then transferred onto a separating gel in slab format with 12% polyacrylamide, and a low molecular weight protein marker (6.5-66.0 kDa) was used as the standard. The gels were run at 120 V and finally stained with Coomassie Brilliant Blue R-250 (CBB R-250, Merck, 1990).

Mass spectrometry and internal sequence analysis.

Enzyme digest of 2-DE SDS-PAGE gels was digested with trypsin and extracted according to the procedures described by Fernandez et al. (1998). Extracted peptides were dried and solubilized in 10 ml of 0.1% TFA. One ml of the 10 μl preparation was used for mass spectrometry analysis, and the remaining 9 ml went to protein purification.

Mass spectrometry analysis was performed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS) technique. One ml of the preparation was deposited on the sample probe of a Voyager Elite mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction and reflection modes. The spectra were internally calibrated using the matrix dimmer (379.05, 1227.15, and 1341.46; Fernandez et al., 1998). Peptide masses were used for tentative protein identification via a peptide mass database search (Protein Prospector; http://www.prospector.ucsf.edu).

The remaining 9 ml of the preparation was purified by high-performance liquid chromatography (HPLC) technique. One of the HPLC purified peptides was determined as a standard for determining amino acid (AA) internal sequence. The internal sequence was obtained through Edman sequence analysis (Fernandez et al., 1998), and it could be used for definitive protein identification via a primary sequence database search (BLAST; http://www.ncbi.nlm.nih.gov).

Western blotting.

Equal amounts of control and treated crude-ESM extract-proteins were separated in one-dimensional (1-DE) SDS-PAGE and were then transferred onto PVDF membrane. Non-specific antibody binding to the PVDF membrane was blocked with 1% BSA in TBS (10 mmol Tris HCl, pH 7.5, 150 mmol NaCl, 0.02% Tween-20) at room temperature for 1 h (Chen et al., 2009). The blot was then incubated using an antibody against the 67 kDa LBP (Elze et al., 1998) at dilution 1:1000 as the primary antibody. Antigen-antibody reaction was detected by anti mouse alkaline phosphatase conjugate (Staph) at dilution 1:500 as the secondary antibody (Dako et al., 1990).

RESULTS

Both control and treated forelimb buds were collected from each of 65 pregnant mice. The gained 2-DE gels indicate that the appearance level of a protein with 41.6 kDa and pI 6.4 increases significantly in the forelimb buds after MAA treatment (Fig. 1), which was obtained from 10 replicates. This significant difference of protein pattern between the control and treated groups was used as consideration for determining that the protein content might be influenced by MAA treatment. Further protein characterization was performed using MALDI TOF-MS and AA internal sequence analysis.

MALDI TOF-MS spectrums were obtained from tryptic digestion of the protein with 41.6 kDa and pI 6.4 (Fig. 2). Twenty-seven peptide masses are applied for tentative protein identification via a peptide mass database search by the ProFound program. The search result details reveal that AA sequence of the matched peptide masses cover 28% of the protein sequence, and that the protein is ribosomal protein RS-40 K (Table 1).

![View larger version](http://www.oxfordjournals.org/content/75/1/148.long)

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<th>Amino Acid Sequence of Matched Peptides from Tryptic Digestion of the Protein with 41.6 kDa and pI 6.4, and a Candidate for the Protein According to the ProFound Search Results</th>
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<td>FIG. 2. MALDI TOF-MS spectrums obtained from tryptic digestion of the protein with 41.6 kDa and pI 6.4. Stars show matched ions identified by ProFound program, and the rest are unmatched peptides.</td>
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For definitive protein identification, the digested protein mixture was purified using HPLC technique. One of the HPLC purified peptides was sequenced using Edman microsequence at 12 cycles. The result reveals KDNTKRNFL as internal sequence. For definitive protein identification, this internal sequence was compared with the primary sequence database (RincB) using the BLAST program. The definitive identification indicates that the protein with 41.6 kDa and pI 6.4 matches exactly to ribosomal protein RS-40 K (Table 2; Fig. 3), which contains 295 AA.

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DISCUSSION

The present study, using a proteomics approach, reveals that the protein with 41.6 kDa and pI of 6.4 is significantly decreased at 4 h after MAA treatment. Definitive protein identification indicates that the protein matches exactly to villin-like protein 2/I-AgRP (Vil-AgRP) receptor, A32335, NCBI Table 2, Fig. 3) and is 95.3% homologous to LBP-β6 (PI-4206, SwissProt). It is known that the LBP is encoded by p40 gene at 71.0 centiMorgans of chromosome 9 in the mouse (MWG 10536; 142). Furthermore, Western blotting with the antibody against the 67 kDa LBP indicates that MAA treatment to pregnant mice causes a decrease of the LBP expression (42 kDa) in the fetal limb buds (Fig. 1), which was obtained from five replicates.

Previous investigations indicate that MAA treatment may interfere with the availability of purine and pyrimidine bases, which are expected to affect DNA and/or RNA synthesis, which in turn influence normal cellular proliferation and differentiation in the developing mouse embryos (Bolton and Wolchik, 1959). Furthermore, the inhibition of RNA or protein synthesis in certain cells may increase apoptotic cell death (Bilbo et al., 1991; Osaki et al., 1998). It is well established that DNA fragmentation is the preliminary event before apoptotic cell death (Bolbo and Ullman, 1998; Olivera et al., 1993). Lmb1 transgenic studies show that the MAA-induced limb abnormalities are caused mainly by intensive cell death in the mesoderm of the limb plates (Rajal et al., 1994; Suda et al., 1995; Sertore et al., 1996).

Moreover, Suda et al. (1995) report that AER also plays a role in the development of the MAA-induced limb abnormality, because it regresses rapidly or treated limbs compared to the control. Recent histological examinations clarify that the MAA-induced cell deaths are apoptotic and apoptosis is predominant than necrosis (Maruyama and Naramasa, 2002).

Kamada et al. (1998), as well as Sato et al. (1994), have clarified that LBP-β6 is associated with both the nuclear envelope and chromatin DNA in interphase nuclei, while it is bound only to chromatin DNA in mitosis. Kamada et al. (1998) state that association of LBP-β6 with histones H2A, H2B, and H4 confers tight binding of LBP-β6 to chromatin DNA in the nucleus. Chromatin DNA may become sensible to the loss of LBP-β6 as a chromatin anchoring protein. Kamada et al. (1998) suggest that the loss of LBP-β6 can induce apoptosis in Hela cells. Therefore, MAA treatment that causes the decline of LBP-β6 production can lead to DNA fragmentation and then induce intensive apoptotic cell death in the mesoderm of the limb plate.

Acht and Brummelen (1992) state that LBP-β6 from mouse cells is associated with histones and polyamines. According to Sato et al. (1995), the localization of LBP-β6 on the 405-nucleosome is well conserved in a broad range of eukaryotes. As a part of the 405 nucleosomes, LBP-β6 is one of the key components of the protein synthetic machinery (Kamada, 2001). The decrease of LBP-β6 in this experiment is predicted to inhibit protein synthesis and induce the intensive apoptotic cell death in the mouse fetial buds. Furthermore, LBP-β6 is considered to be a precursor or a basic building block of the 67 kDa LBP (Sato et al., 1993; Nara et al., 1999; Sato et al., 1999; Tanioka et al., 2000; Verbeet et al., 2003). Lumbosst et al. (1995) indicate that the 67 kDa LBP might be a dimer of LBP-β6, and that ablation may occur before or after dimersization. Meanwhile, Wang et al. (1995) report that overproduction of LBP-β6 in 405 cells increases the production of the 67 kDa LBP at the cell surface.

In the early stage of chick limb development, a 67 kDa lamin-binding protein gene (LBP-β6) is expressed in the mesodermal region of the limb plate and in the AER. The 67 kDa LBP plays a role in maintaining the Fc mesoderm in an uncommitted state during the development of the chick limbs (Peirce et al., 1973). This protein might serve as an auxiliary molecule involved in regulating the interaction between the endoderm and mesoderm integrins (Kamada, 2001). It is suggested that Fc and P integrins are required for the organization of a common pool of presumptive AER cells into a distinct differentiated structure (Macara et al., 1990). The facts indicate that the 67 kDa LBP is required for the normal growth of AER. Therefore, AER treatment that decreases the LBP expression may decrease the 67 kDa LBP production in the AER. The decline of LBP-β6 expression indirectly causes the degradation of the AER in the limb buds. Suda et al. (1995) report that the AER regression rapidly on the MAA-treated limbs compared to the control. The alteration of AER properties lead to reduction in the proliferation of the underlying mesenchymal cells.

It can be concluded that the decline of LBP-β6 expression after MAA treatment stimulates both the intensive apoptotic cell deaths in the mesoderm of the limb plate and the AER regression, which induces limb abnormality of the mouse fetuses.

Acknowledgments

A Graduate Team Research Grant, University Research for Graduate Education (URGE) Project Batch III, supported this study. Protein sequence analysis was provided by the Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment, and with the help of Joseph Fernandez.

Footnotes

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The Lammin Binding Protein p40 Is Involved in Inducing Lymphomas...

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The Lamactin Binding Protein p40 Is Involved in Inducing Limb Abnormalities

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93-99.

Recent studies have shown that the lamactin binding protein (LBP) p40 is involved in inducing limb abnormalities. LBP p40 is encoded by the lamactin gene and is expressed in developing limb bud mesenchyme. The LBP p40 protein binds to the lamactin mRNA, and this binding is thought to be mediated by an RNA-binding protein (RBP). The interaction between LBP p40 and lamactin mRNA is thought to be required for normal limb development.


These findings suggest that the LBP p40 protein plays a critical role in the regulation of limb development. Further studies are needed to elucidate the molecular mechanisms underlying this process.