Developmental alterations in chick embryo by β-microseminoprotein are closely associated with modulation of goosecoid and noggin expression

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β-Microseminoprotein (MSP)-induced enhancement of mesodermal structures in chick embryo is brought about through modulation of expression of Brachyury and is often associated with abnormal nervous system development. In the present study, carried out to further elucidate the mechanism of action of MSP, we find that treatment with MSP leads to up-regulation of goosecoid and down-regulation of noggin in cultured chick embryos. These observations correlate well with stimulation of axis elongation and interference with neural tube pattern formation due to MSP. The results show that the molecular mechanism of action of MSP involves goosecoid and noggin, in addition to Brachyury, and further support our contention that MSP-related molecules play an important role in the embryonic development of chick.

HUMAN β-microseminoprotein (MSP; also known as human seminal plasma inhibin, hSP-I or prostatic secretory protein of 94 amino acid, PSP94) brings about enhancement in the development of mesodermal structures in the chick embryo explants cultured in vitro. We have recently shown that this action of MSP is exerted through modulation of expression of Brachyury. The product of Brachyury is a transcription factor, which interacts with a number of downstream genes leading to the formation of mesoderm and gastrulation movements.

MSP-induced enhancement of development of mesodermal structures in chick embryos is often associated with abnormal development of neural structures. MSP also leads to elongation of the anteroposterior axis and increased cellular movements through the Hensen’s node. An important protein that controls cell migration during gastrulation is the product of goosecoid. This is a homeobox gene that is expressed in the organizer region during gastrulation of Xenopus, chick and mouse. Brachyury has been shown to interact with goosecoid in Xenopus. Morphological changes and modulation of Brachyury in developing chick embryo due to MSP suggest that the latter may exert its effects through modulation of goosecoid, either directly or indirectly.

A prominent effect of MSP in the chick embryo is abnormalities in the patterning of the nervous system. We therefore studied the expression of noggin, a gene important in neural tube patterning in chick. In Xenopus, Noggin is one of the neural inducers and its neural inducing action is the result of its capacity to neutralize BMP4. In the chick embryo, noggin is expressed at sites comparable to those in Xenopus, but it does not appear to function as a neural inducer. Misexpression of noggin does not lead to formation of neural plate at ectopic sites in chick. Noggin is therefore believed to participate in the patterning of the neural tube in chick.

In view of the effects of MSP on the development of axial structures, neural patterning, morphogenetic movements and expression of Brachyury in chick embryo, the present study was undertaken to analyse the influence of MSP on the expression of goosecoid and noggin. The results show that MSP differentially modulates the expression of these two developmentally crucial genes and further support our hypothesis that MSP-related molecules play an important role in early chick embryonic development.

Materials and methods

Materials

Freshly laid White Leghorn chicken eggs were obtained either from Central Hatchery, Pune or Institute of Veterinary Biological Products, Pune.

In vitro culture, staging and treatment of chick embryo explants: The eggs were incubated at 37.5°C for required duration to obtain the desired stages of development. Chick embryos were dissected in Pannet–Compton saline (PC saline, pH 7.4) and cultured in vitro using New’s single ring technique. The embryos were staged on the basis of morphological criteria. Gastrulating chick embryos of Hamburger–Hamilton (HH) stages 3, 4, 5, 8 and 11 were used in the present study. Embryos were treated as described earlier. One hundred microlitres of PC saline containing MSP (kind gift from S. R. Moodbidri, NIRRH, Mumbai)

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was carefully placed on the embryo, inside the ring to the final concentration of 9.5 nM. The embryos were left at room temperature for 30 min to allow diffusion of MSP and then incubated at 37.5°C for 2 h. Effect of MSP on the expression of goosecoid was studied at HH stages 3, 4 and 5, while that on the expression of noggin was studied at HH stages 4, 5, 8 and 11.

Study of goosecoid and noggin expression by whole mount in situ hybridization: Plasmid pGsc2 harbouring a 367 bp fragment inclusive of the 5' end of the Xenopus goosecoid cDNA cloned into a pBluescript KS vector (kind gift from Jim Smith, Cambridge, UK) was linearized with XbaI and transcribed using T3 RNA polymerase in the presence of DIG labelled dUTPs (Roche, Germany) to get an antisense transcript of 440 bases. Plasmid clone of noggin was a kind gift from Jim Smith. Full length noggin cDNA was cloned by deleting 857 bp from the 3' end within the EcoRV site. Clone npnoggin5.5 was linearized with EcoRI and transcribed using T7 RNA polymerase in the presence of DIG labelled dUTPs to get an antisense transcript of 463 bases.

Whole mount in situ hybridization was performed according to Nieto et al.\textsuperscript{18}, with a few modifications\textsuperscript{2,19}. The in situ hybridized embryos were refixed in 4% paraformaldehyde in PBS overnight at 4°C, washed in PBS, dehydrated in methanol for 5 min, transferred to isopropanol for 10 min and cleared in benzene for 15 min. These were embedded in paraffin wax with cerasin and transverse sectioned at 15 μm thickness with a Bright (UK) rotary resecting microtome. The sections were dewaxed and cell type-specific expression of goosecoid and noggin was studied.

Northern blotting: RNA was extracted using TRIZOL reagent (Gibco, BRL).\textsuperscript{20,21} Northern hybridization was carried out as described earlier. Quantitative analysis of relative abundance of transcripts was carried out using Herolab Easywin software (Germany).

Results

Effect of MSP on goosecoid expression

The effects of MSP on the expression of goosecoid were studied at HH stages 3, 4 and 5 by whole mount in situ and Northern hybridization.

Stage 3: Goosecoid expression was seen in the anterior three fourth of the elongating streak. The intensity of staining was more in the anterior tip than in the remaining streak (Figure 1a). In MSP-treated embryos, goosecoid was expressed all over the streak and in a relatively broader area (Figure 1b). The overall intensity of staining was also higher in MSP-treated embryos. The effect was evident in 100% of treated embryos (Table 1). This up-regulation of goosecoid expression observed in whole mount in situ

![Figure 1. Effect of β-microseminoprotein (MSP) on goosecoid expression. Chick embryos were explanted at HH stages 3, 4 or 5 and cultured in the presence or absence of 9.5 nM MSP for 2 h. Goosecoid transcripts were detected by whole mount in situ hybridization using an antisense riboprobe. a, Control stage-3 embryo showing expression of goosecoid in cells of the primitive streak. Note more intense staining in the anterior tip of the primitive streak (arrowhead). b, MSP-treated embryo showing goosecoid expression all over the streak and in a broader area than in control. Note overall higher staining intensity. c, Control stage-4 embryo showing expression of goosecoid in cells of Hensen’s node (arrow). d, MSP-treated stage-4 embryo showing more intense staining for goosecoid in Hensen’s node. In addition, goosecoid was also expressed throughout the primitive streak in these embryos (arrowheads). e, Transverse section of a control embryo at the level indicated in (c). Goosecoid is localized to the ingressing cells. f, Transverse section of MSP-treated embryo at a comparable level to that indicated in (d). Note significantly high level of goosecoid expression in the ingressing cells. g, Control stage-5 embryo with goosecoid expression in cells of the primitive streak, newly formed prechordal plate and prospective neural tube. h, MSP-treated stage-5 embryo showing elevated goosecoid expression in cells of the primitive streak, prechordal plate and prospective neural plate. Note that the regressing Hensen’s node continues to express goosecoid. i, (Upper panel) Northern blot for detection of goosecoid transcripts in control (C) and treated (T) embryos of stages 3, 4 and 5. (Lower panel) Ethidium bromide-stained RNA from chick embryos. Intensities of signal on Northern blot were normalized against those of 28S and 18S bands. Quantitative analysis of relative abundance of transcripts was carried out from Northern blots using Herolab Easywin software (Herolab, Wiesloch, Germany). j, Histogram depicting relative abundance of goosecoid transcripts in control embryos and embryos treated with MSP at various stages of development.]
Table 1. Effect of β-microseminoprotein on expression of goosecoid and noggin in chick embryo explants cultured in vitro

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Control</th>
<th>MSP (9.5 nM)-treated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of embryos</td>
<td>Expression</td>
</tr>
<tr>
<td></td>
<td>Normal (%)</td>
<td>Modified (%)</td>
</tr>
<tr>
<td>Goosecoid</td>
<td>HH stage 3</td>
<td>24 (100)</td>
</tr>
<tr>
<td></td>
<td>HH stage 4</td>
<td>32 (100)</td>
</tr>
<tr>
<td></td>
<td>HH stage 5</td>
<td>28 (100)</td>
</tr>
<tr>
<td>Noggin</td>
<td>HH stage 4</td>
<td>09 (100)</td>
</tr>
<tr>
<td></td>
<td>HH stage 5</td>
<td>30 (100)</td>
</tr>
<tr>
<td></td>
<td>HH stage 11</td>
<td>19 (100)</td>
</tr>
</tbody>
</table>

*At HH stage 4, the expression of noggin was at barely detectable levels by in situ hybridization making it difficult to assess difference in its expression in control and treated embryos (for details, see text).

hybridization was clearly reflected in Northern hybridization. The relative abundance of goosecoid transcripts in these embryos was 48% higher than in controls (Figure 1 i, j).

Stage 4: At HH stage 4, goosecoid transcripts were localized to the Hensen’s node in control embryos (Figure 1 c). In all the MSP-treated embryos, once again, goosecoid expression was up-regulated (Table 1). In addition to the more intense staining in cells of the Hensen’s node, these embryos expressed goosecoid throughout the primitive streak (Figure 1 d). Significant increase in staining intensity due to MSP treatment was clearly evident in transverse sections of control and treated embryos (Figure 1 e, f). When relative abundance of transcripts was studied by Northern blotting, MSP-treated embryos showed an overall increase of 60% in the abundance of goosecoid transcripts (Figure 1 i, j).

Stage 5: At this stage, control embryos showed expression of goosecoid in cells of the primitive streak, newly formed prechordal plate and prospective neural plate (Figure 1 g). In MSP-treated embryos, goosecoid was expressed in cells of the regressing node. Additionally, staining intensity was more in primitive streak, prechordal plate and prospective neural plate (Figure 1 h). However, unlike stages 3 and 4 embryos, only about half of the embryos treated at stage 5 responded to MSP treatment in this manner (Table 1). In any case, the increase in goosecoid expression in MSP-treated stage-5 embryos was significant, as seen from the relative abundance of transcripts in control and treated embryos (Figure 1 i, j).

Thus, in all the three stages studied, HH stages 3, 4 and 5, MSP treatment led to up-regulation of goosecoid that was evident both in whole mount in situ and Northern hybridization. The up-regulation was maximum at stage 4. At stage 5, response of goosecoid to MSP seemed to decline, as seen from reduction in the proportion of responding embryos.

Effect of MSP on noggin expression

Effect of MSP on the expression of noggin was studied at HH stages 4, 5, 8 and 11 using whole mount in situ and Northern hybridization.

Stage 4: At this stage, noggin was expressed around the primitive streak. The level of expression, however, was very low (data not shown). In MSP-treated embryos, the expression was marginally above the level of detection by whole mount in situ hybridization (data not shown). As a result of such low abundance of noggin transcripts, detection of alterations in noggin expression, if any, was not possible. On the other hand, in the Northern analysis, a substantial 25% decrease in the abundance of noggin due to MSP treatment was evident (Figure 2 k, l).

Stage 5: The level of expression of noggin at stage 5 was much higher than at stage 4. In controls, noggin transcripts were localized to the newly formed notochord and the prospective neural plate (Figure 2 a). MSP-treated embryos exhibited expression of noggin in cells of an identical area, but the staining intensity was greatly reduced (Figure 2 b). All the MSP-treated embryos showed down-regulation of noggin (Table 1). This response to MSP was also clearly reflected in the relative abundance of noggin transcripts (Figure 2 k, l).

Stage 8: At stage 8, noggin expression was localized to almost the entire length of the neural folds (Figure 2 c). In controls, the staining was more intense in the anterior one-third of the neural folds and maximum in the midbrain–hindbrain boundary region (Figure 2 c). MSP treatment led to down-regulation of noggin, resulting in considerably reduced staining intensity (Figure 2 d). All the treated embryos showed this response.

Stage 11: Control embryos of stage 11 showed expression of noggin in cells of the neural tube. A high expression level
Figure 2. Effect of MSP on noggin expression. Chick embryos were explanted at HH stages 5, 8 or 11 and cultured in the presence or absence of 9.5 nM MSP for 2 h. Noggin transcripts were detected by whole mount in situ hybridization using an antisense riboprobe. a, Control stage-5 embryo showing noggin expression in cells of the prospective neural plate and anterior portion of the notochord. The newly formed notochordal tissue lacks the expression of noggin. b, MSP-treated stage-5 embryo showing similar pattern of expression of noggin; the intensity of staining however is greatly reduced. c, Control stage-8 embryo showing noggin expression throughout the length of the neural folds. Note that transcripts are more abundant in the anterior one-third of the neural folds (arrowheads) and maximum at midbrain–hindbrain junction (arrow). d, MSP-treated stage-8 embryo with significantly reduced expression of noggin. e, Stage 11 control embryos showing intense staining for noggin in the brain region of the neural tube. The midbrain–hindbrain boundary is marked with maximum expression of noggin (arrowhead). Note that noggin is also expressed in the somites, although at very low levels (arrows). f, MSP-treated stage 11 embryo showing presence of noggin transcripts in identical embryonic areas, but in greatly reduced numbers leading to faint staining. g, Transverse section of stage-11 embryo passing through brain region at the level indicated in (e). The neural tube, foregut and somatic mesoderm are seen. Expression of noggin is localized to the cells of the neural tube. h, Transverse section of MSP-treated stage-11 embryo passing through a comparable region at the level shown in (f). i, Histogram depicting relative abundance of noggin transcripts in control embryos and those treated with MSP at various stages of development.

was seen in the region of the midbrain–hindbrain boundary (Figure 2e). Noggin was also expressed in the somites. MSP treatment led to an overall down-regulation of noggin (Figure 2f). This rather drastic reduction of noggin expression was clearly visible in the transverse sections on in situ hybridized embryos (Figure 2g–j). Noggin expression in the neural tube as well as in the paraxial mesoderm (Figure 2i, j) was down-regulated as a result of treatment with MSP.
Northern analysis confirmed the effect of MSP on noggin expression (Figure 2k, l). All the treated stage-11 embryos exhibited this response (Table 1).

Discussion

Our earlier work has shown that treatment of developing chick embryos with human MSP leads to enhancement of development of mesodermal structures, elongation of body axis and abnormalities in the nervous system. One of the molecular pathways through which MSP appears to exert its effects involves Brachyury, the product of which is a transcription factor crucial for the development of the mesoderm. However, the spectrum of developmental alterations brought about by MSP in the chick embryo cannot be fully explained merely on the basis of modulation of Brachyury expression by MSP. We have therefore carried out further studies on the effects of MSP on the expression of two more developmentally important genes. These are goosecoid and noggin that play crucial roles during early embryonic development and pattern formation of the chick embryo. From our earlier studies, we know that human MSP brings about the most effective enhancement of mesodermal structures in chick embryo at the concentration of 9.5 nM. The same concentration has been used in this study. We have indeed demonstrated the presence of MSP-like molecules in chick embryo; however, MSP has not yet been isolated from chick embryo. Due to this reason we have continued to use human MSP for further studies.

Up-regulation of goosecoid by MSP treatment

Treatment of developing chick embryo explants with MSP resulted in an up-regulation of goosecoid within 2 h as detected by whole mount in situ and Northern hybridization, at all the three developmental stages used. The effect, however, was not identical at all stages; it was less prominent at stages 3 and 5 than at stage 4 in terms of intensity of staining, indicating abundance of transcripts. There was also a difference in the proportion of embryos responding to MSP at different stages. At stages 3 and 4, 100% treated embryos showed increased goosecoid. At stage 5, the response was evident only in about 50% of the treated embryos. Thus, the proportion of embryonic cells capable of responding to MSP starts reducing once the primitive streak has attained its maximum length.

Gsc is a homeodomain protein that acts as a transcription factor. There is an extensive homology between Gsc proteins and also goosecoid gene sequences from a variety of vertebrates like Xenopus, chick and mouse. When mRNA for chick goosecoid is injected in Xenopus embryos at 4-cell or 8-cell stages, it can induce a secondary axis. We found that MSP treatment upregulated goosecoid at stages 3, 4 and 5. At stage 3, considerable cellular movements are in progress and the epiblast cells are undergoing convergent extension. As a consequence, the primitive streak lengthens. Increased goosecoid expression at this stage may lead to increased cell movements since goosecoid-expressing cells participate in gastrulation movements. Increased goosecoid expression also indicates a better potential to induce axial structures.

At stage 4, goosecoid is up-regulated to an even greater extent due to MSP treatment. The anterior one-third of the primitive streak in treated embryos showed goosecoid expression as against restricted expression in the Hensen’s node in control embryos. These additional goosecoid-expressing cells in treated embryos are likely to have acquired organizing and neural inducing abilities. Increased goosecoid expression may also be responsible for MSP-induced enhanced movement of cells through the anterior portion of the streak reported recently. Expression of goosecoid by additional cells may also divert them to form the prechordal plate, since cells that express goosecoid are destined to form the prechordal plate. Indeed, the enhancement of notochordal length after treatment with MSP, evident in gross morphological studies, may have resulted from this.

In stage-5 embryo treated with MSP for 2 h, goosecoid expression appeared to persist in the regressing Hensen’s node, in addition to the prechordal plate and prospective neural plate. Continued expression of goosecoid in the node would be expected to confer on it a sustained organizer function. It is known that goosecoid-expressing cells are destined to become mesodermal and possess neural inducing ability. Continued organizer function in the Hensen’s node in MSP-treated stage-5 embryos thus may have directed additional cells to become mesodermal and also induced neural tissue to a greater extent.

In Xenopus and mouse, an interaction between Brachyury and goosecoid has been demonstrated. Gsc suppresses Brachyury in both these species. However, we did not detect any suppression of Brachyury expression as a result of increase in goosecoid expression in our earlier or present study. This is probably because the expression of both Brachyury and goosecoid was studied at only 2 h treatment, which may not be sufficient for the Gsc protein to be synthesized and exert its effect on Brachyury expression.

Down-regulation of noggin by MSP treatment

Noggin encodes a secreted factor. In Xenopus, Noggin acts as one of the neural inducers by binding to BMP4 and inhibiting it from binding to its own receptor. In the chick embryo, however, the role of Noggin is different than in the Xenopus embryo. Although the site of expression of noggin is comparable in both, noggin is detectable in the Hensen’s node only after the neural inducing ability of the node starts diminishing. Further, ectopic expression of noggin does not induce a neural fate in competent ectodermal cells in the chick embryo. Absence of Noggin protein, however, leads...
to patterning defects in the neural tube in mouse. In view of the role of noggin in the patterning of neural tube in chick, we have studied the effects of MSP on noggin expression from stages 4 through 11. At stage 4, there exists only the prospective neural plate, while by stage 11 the neural tube pattern is well evident.

At all the stages studied, viz. stages 4, 5, 8 and 11, MSP down-regulated noggin. At stage 4, due to barely detectable noggin expression in whole mount in situ hybridization, the effect was evident only in the Northern analysis. At stage 5, noggin transcripts were localized to the prospective neural plate and anterior-most part of the notochord. The abundance of noggin transcripts was significantly reduced due to MSP treatment, which may have affected the future patterning events. At stages 8 and 11, when neurulation and pattern formation of the neural tube are in progress, noggin expression was once again found to be down-regulated by MSP. This would mean reduced amounts of Noggin protein available for participation in proper progression of neurulation, formation of brain compartments and dorsoventral patterning of the neural tube. Thus, reduced amounts of noggin transcripts in MSP-treated embryos at developmental stages 4, 5, 8 and 11, when crucial steps in neurulation are in progress, appear to be the underlying cause for the development of abnormal nervous system in such embryos at the end of a 22 h treatment.

**Summary and conclusion**

Effects of human MSP at gross morphological level include enhancement of mesodermal structures, elongation of the anteroposterior body axis and abnormalities in the nervous system. Up-regulation of goosecoid and down-regulation of noggin at relevant stages of development by MSP appear to be the underlying causes for axis elongation and abnormal nervous system respectively. MSP has the capacity to simultaneously alter the expression pattern of all the three important genes studied so far. Human MSP modulates the distribution of available Brachyury transcripts, up-regulates goosecoid (present study), and down-regulates noggin (present study), in chick embryo explants cultured in vitro within 2 h. These specific effects of MSP at the molecular level lead to significant alterations in the embryonic development and pattern formation in the chick embryo (Figure 3). We have recently shown that chick seminal fluid and developing chick embryos contain MSP-related molecules. Taken together, our studies demonstrate that MSP-related molecules play a crucial role in the early embryonic development and pattern formation in the chick embryo.

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