Distribution and possible function of an Adrenomedullin-like peptide in the developing chick limb bud

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ABSTRACT   Adrenomedullin (AM) is a multifunctional peptide that exhibits discrete domains of expression during mouse embryogenesis consistent with a role in regulating growth and differentiation during morphogenesis. Here we report that AM immunoreactivity is present at high levels throughout the apical ectodermal ridge (AER) of the chick limb bud as the AER is directing the outgrowth and patterning of underlying limb mesoderm. Immunostaining is particularly strong along the surfaces of the contiguous cells of the AER. AM immunoreactivity attenuates as the AER regresses and is absent from the distal apical ectoderm of stage 20 *limbless* mutant limb buds which fail to develop an AER. To explore the possible role of AM in AER activity, we examined the effect of exogenous AM and an AM inhibitor on the *in vitro* morphogenesis of limb mesoderm, cultured in the presence and absence of the AER. Although exogenous AM cannot substitute for the AER in promoting outgrowth of limb mesoderm *in vitro*, a specific AM antagonist, AM(22-52), impairs the outgrowth and proliferation of limb mesoderm cultured in the presence of the AER. This is consistent with the possibility that inhibition of endogenous AM activity in the AER impairs the ability of the AER to promote limb morphogenesis. Taken together, these studies suggest that an AM-like molecule may function in an autocrine fashion to regulate some aspect of AER activity.

KEY WORDS: *adrenomedullin, limb development, AER, limbless mutant chick embryos, proliferation*

Introduction

Adrenomedullin (AM) is a 52 amino acid multifunctional peptide that is conserved across many species, and has been implicated in regulating a variety of physiological processes (Kitamura *et al.,* 1993a,b, 1994; Sakata *et al.,* 1993; Martínez *et al.,* 1995, 1996; Martínez and Cuttitta, 1998). Studies have suggested that AM can function as an autocrine or paracrine regulator of growth and proliferation (Withers *et al.,* 1996; Miller *et al.,* 1996; Martínez and Cuttitta, 1998). AM is highly expressed by numerous malignant cell lines, and a neutralizing anti-AM monoclonal antibody inhibits tumor cell growth (Miller *et al.,* 1996).

It has recently been demonstrated that AM exhibits discrete spatial and temporal patterns of expression during mouse and rat embryogenesis that are consistent with its possible involvement in regulating growth and differentiation during morphogenesis (Montuenga *et al.,* 1997, 1998). Notably, AM is localized at several sites of epithelial-mesenchymal interactions in the developing embryo, suggesting a possible role in inductive tissue interactions (Montuenga *et al.,* 1997, 1998).

These observations prompted us to explore the expression and possible function of AM during the development of the chick limb bud which provides an excellent and readily manipulable model system for investigating the roles of growth factors and signaling molecules in tissue interactions. The outgrowth and patterning of the developing chick limb bud is dependent on reciprocal interactions between a thickened cap of ectoderm at its distal tip called the apical ectodermal ridge (AER) and the underlying mesoderm (Saunders, 1977). The AER promotes the proliferation and outgrowth of the subridge mesoderm (Saunders, 1948), while maintaining it in a labile undifferentiated condition (Kosher *et al.,* 1979; Solursh *et al.,* 1981). The AER also maintains the activity of the polarizing zone at the posterior margin of the limb bud (Vogel and Tickle, 1993), which, in turn, appears to be the source of a morphogen that regulates pattern formation along the anteroposterior axis of the limb bud (Saunders and Gasseling, 1968). Here we report that AM immunoreactivity is

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Abbreviations used in this paper: AER, apical ectodermal ridge; AM, adrenomedullin; BrdU, 5-bromodeoxyuridine; FGF, fibroblast growth factor; IGF, insulin-like growth factor.

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present in high amounts in the AER as the AER is promoting the outgrowth and patterning of limb mesoderm. We also provide evidence suggesting that an AM-like molecule may function in an autocrine fashion to regulate some aspect of AER activity.

Results

Distribution of AM Immunoreactivity during Early Development of the Chick Limb Bud and in Limbless Mutant Limb Buds

From stage 18, which is shortly after the formation of the limb bud, through stage 25, a high level of AM immunoreactivity is present throughout the AER at the distal periphery of the limb bud, which is directing the outgrowth and patterning of the underlying limb mesoderm (Fig. 1 A-E). The intensity of immunostaining in the AER is highest during the early stages of limb development (Fig. 1 A-D) and attenuates by stage 25 (Fig. 1E). Little immunostaining is detectable in the distal apical ectoderm by stage 31, by which time the AER has lost its ability to promote the outgrowth of limb mesoderm (Rubin and Saunders, 1972). At least at early stages of
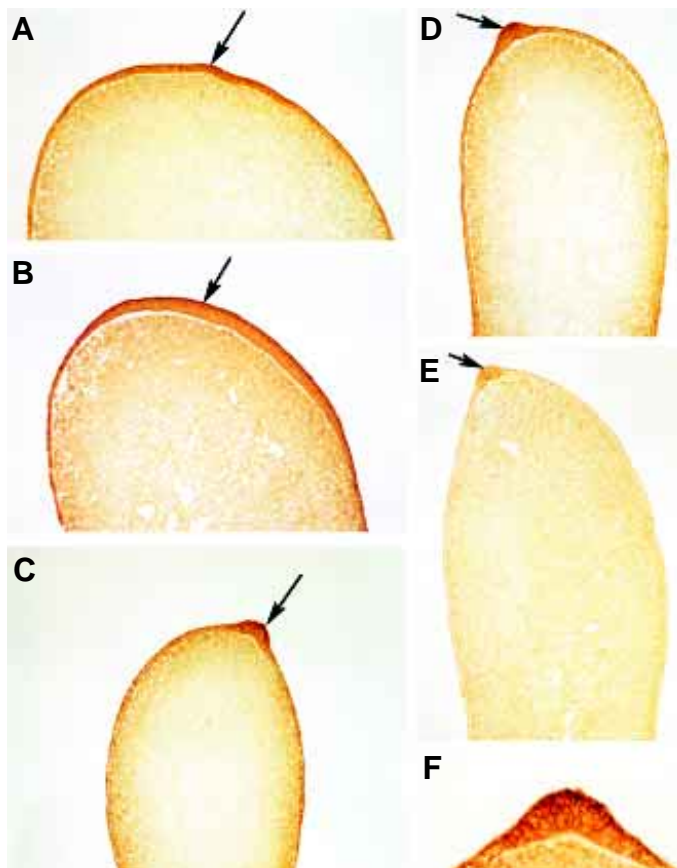
Fig. 3. Day 4 control (A) and 10⁻⁶ M AM-treated (B) posterior limb mesoderm explants *cultured in the absence of the AER and limb ectoderm. Exogenous AM does not promote the outgrowth of the explants.*

development, weak immunostaining is detectable in dorsal/ventral limb ectoderm and perhaps in mesodermal cells directly subjacent to the ectoderm (Fig. 1 A-D). However, immunostaining in these regions is very modest compared to the intense AM immunoreactivity present in the AER. It is noteworthy that very intense immunostaining is present along the surfaces of the contiguous cells of the AER (Fig. 1F).

To explore further the association of AM with AER activity, we examined its distribution during the development of the limb buds of *limbless* mutant chick embryos. *Limbless* is an autosomal recessive mutation that directly affects the ectoderm, not the mesoderm, of limb buds (Carrington and Fallon, 1988). Limb buds of *limbless* mutant embryos form at the proper time in development, but fail to undergo further outgrowth and subsequently degenerate (Carrington and Fallon, 1988). The lack of outgrowth of *limbless* limb buds results from their failure to form an AER (Carrington and Fallon, 1988), and this is reflected in the absence of expression of a variety of AER-characteristic genes in *limbless* apical ectoderm (Ros *et al.,* 1996; Grieshammer *et al.,* 1996; Coelho *et al.,* 1991; Robert *et al.,* 1991).

AM immunoreactivity is present in the ectoderm during the initial formation of *limbless* mutant limb buds at stage 17 (Fig. 2B), which is an event that occurs prior to and independent of the formation of the AER (Carrington and Fallon, 1988; Todt and Fallon, 1984).

Fig. 1. (Left) Distribution of AM immunoreactivity during early development of the chick limb bud. *Frontal (A,B) and sagittal (C-E) sections through stage 19/20 (A), stage 22 (B,C), stage 23 (D) and stage 25 (E) limb buds. Immunostaining is detectable throughout the AER (arrows). (F) Sagittal section through the AER of a stage 22 limb bud. Intense AM immunostaining is present along the surfaces of the contiguous cells of the AER.*

Fig. 2. (Right) Distribution of AM immunoreactivity during the development of *limbless* mutant limb buds. *(A,C) Frontal sections through stage 18 (A) and stage 20//21 (C) normal (non-mutant) limb buds. AM immunostaining is present throughout the AER of normal limb buds at both stages. (B,D) Frontal sections through stage 17 (B) and stage 20/21 (D)* limbless *mutant limb buds. AM immunostaining is present throughout the apical ectoderm of* limbless *limb buds at stage 17, but not at stage 20/21.*
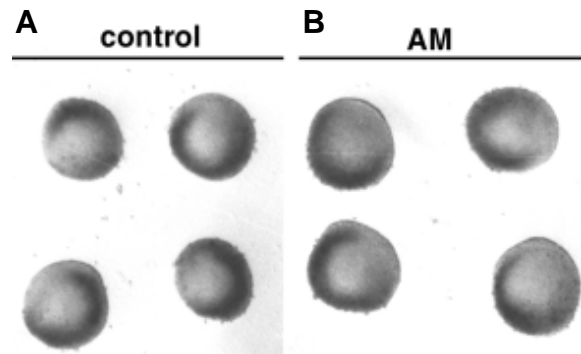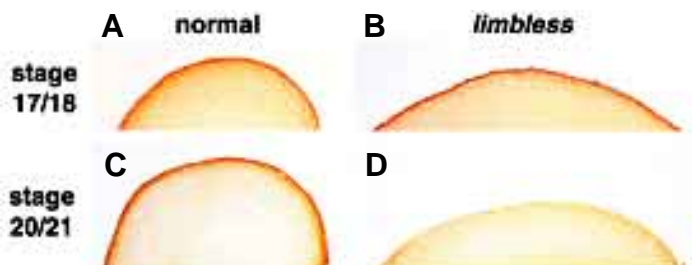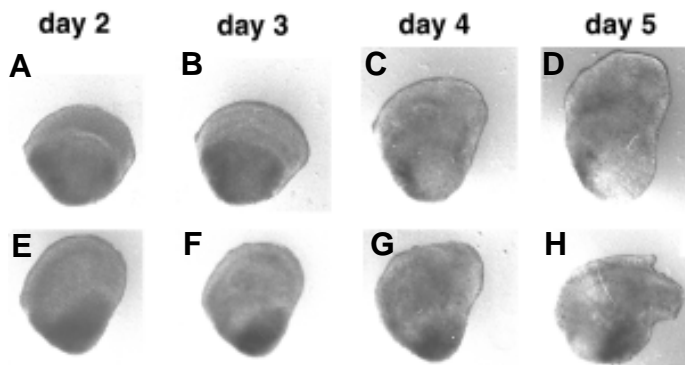
day 2   day 3   day 4   day 5

Fig. 4. Morphogenesis of a control (A-D) and 10⁻⁶ M AM(22-52)-treated (E-H) posterior limb mesoderm explant cultured in the presence of the AER. *The control explant undergoes progressive proximodistal outgrowth during the 5 day culture period, whereas the AM(22-52)-treated explant undergoes little or no outgrowth between days 2 and 5.*

However, as shown in Fig. 2D, the distal apical ectoderm of stage 20 *limbless* mutant limb buds, which have failed to develop an AER, exhibits little or no immunostaining for AM, as might be expected for a molecule involved in AER activity.

In Vitro *Studies of the Possible Role of AM in AER Activity*

The presence of a high level of AM immunoreactivity in the AER as it is directing the outgrowth of limb mesoderm and its absence from the apical ectoderm of stage 20 *limbless* mutant limb buds which fail to develop an AER suggests that an AM-like molecule may play a role in regulating or mediating some aspect of AER activity. One possibility is that AM expressed in the AER may function in a paracrine fashion to mediate the effect of the AER on the outgrowth of limb mesoderm. To explore this possibility, we determined if exogenous AM would be able to promote the outgrowth of posterior limb mesoderm subjected to organ culture in the absence of the AER and limb ectoderm, as do other signaling molecules such as IGF-I and FGFs that have been implicated in mediating the outgrowth of limb mesoderm in response to the AER (Dealy and Kosher, 1995, 1996). As shown in Fig. 3, exogenous AM at concentrations ranging from 10^{-8} M to 10^{-5} M does not promote the outgrowth of posterior limb mesoderm explants lacking an AER.

Another possibility is that AM present in the AER may be regulating in an autocrine fashion some aspect of the activity of the AER itself. Consistent with this possible autocrine mode of action is the observation that a high level of AM immunoreactivity is present along the surfaces of the contiguous cells of the AER (Fig. 1F). If AM is indeed acting in an autocrine fashion to influence AER activity, then inhibiting endogenous AM activity in the AER might impair the ability of the AER to promote the morphogenesis of limb mesoderm. Accordingly, we examined the effect of the AM antagonist AM(22-52)

on the morphogenesis of posterior limb mesoderm explants cultured in the presence of the AER. AM(22-52) contains the C-terminal 30 amino acids of AM which mediate binding of AM to its receptor, but lacks the N-terminal portion of the molecule required for signal transduction following receptor binding (Eguchi *et al.,* 1994).

The gross morphogenesis of control and AM(22-52)-treated posterior limb mesoderm explants cultured in the presence of the AER is shown in Fig. 4. During the 5 day culture period, control explants progressively undergo polarized proximodistal outgrowth (Fig. 4 A-D). The outgrowth and contour of AM(22-52)-treated explants is comparable to controls through day 2 of culture (Fig. 4E). However, in contrast to controls, AM(22-52)-treated explants undergo little or no further outgrowth during the subsequent 3 days of culture (Fig. 4 E-H). The size and shape of day 3-5 AM(22-52)-treated explants is comparable to that of day 2 control and treated explants.

To confirm the observations on living explants indicating that AM(22-52) impairs the outgrowth of limb mesoderm in response to the AER, we compared cell proliferation in control and AM(22-52)-treated explants by immunohistochemical analysis of BrdU incorporation into nuclei engaged in DNA synthesis during a 1 hour labeling period on days 3 and 4 of culture. As shown in Fig. 5, a highly proliferating population of cells is present in the mesoderm underlying the AER of control explants, and there is a striking reduction in number of proliferating cells in the subridge mesoderm of the AM(22-52)-treated explants. The subridge mesoderm of control explants
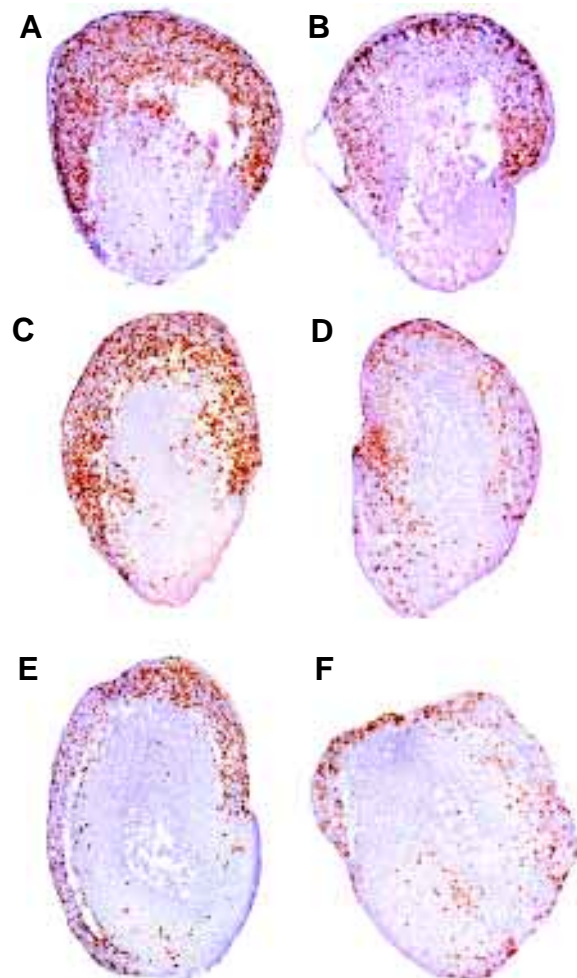

Fig. 5. Cell proliferation in control (A,C,E) and 10⁻⁶ M (B) or 10⁻⁵ M (D,F) AM(22-52)-treated posterior limb mesoderm explants *cultured in the presence of the AER as assayed by the immunohistochemical analysis of BrdU incorporation into dividing cells during a 1 hour labeling period on day 3 (A,B; C,D) or day 4 (E,F) of culture. A highly proliferating population of cells is present in the mesoderm underlying the AER of control explants, and there is a striking reduction in number of proliferating cells in the subridge mesoderm of the AM(22-52)-treated explants.*

contains more than twice as many proliferating (BrdU-labeled) cells as AM(22-52)-treated explants (Table 1). Thus, the outgrowth and proliferation of limb mesoderm in response to the AER is clearly impaired in the presence of AM(22-52).

TABLE 1

EFFECT OF AM(22-52) ON CELL PROLIFERATION IN THE SUBRIDGE MESODERM OF POSTERIOR LIMB MESODERM EX-PLANTS CULTURED IN THE PRESENCE OF THE AER

Number of proliferating cells[a]	
Control explants	AM(22-52)-treated explants
287 ± 29 ($n = 6$)	131 ± 21 ($n = 9$)

[a]The number of BrdU-labeled nuclei in the subridge mesoderm of sections through the center of control and 10^{-5} M AM(22-52)-treated explants that had been incubated in the presence of BrdU for 1 hour on day 4 of culture.

Discussion

AM is a multifunctional peptide that has recently been shown to exhibit discrete spatial and temporal patterns of expression during mouse and rat embryogenesis, particularly at sites of epithelial-mesenchymal tissue interactions (Montuenga *et al.,* 1997, 1998). Here we demonstrate that AM immunoreactivity is present at high levels in the AER during the early stages of development of the chick limb bud when the AER is directing the outgrowth and patterning of limb mesoderm. AM immunoreactivity attenuates during later stages of development as the AER flattens and loses its ability to promote limb outgrowth. Furthermore, little or no AM immunoreactivity is detectable in the apical ectoderm of stage 20 *limbless* mutant limb buds which have failed to develop an AER. These results suggest that an AM-like molecule may play a role in regulating or mediating some aspect of the activity of the AER of the developing chick limb bud.

Our results suggest that an AM-like molecule may function in an autocrine fashion to regulate some aspect of AER activity. Intense immunostaining for AM is present along the surfaces of the cells of the AER, an observation consistent with the possibility that AM expressed by AER cells may be bound to receptors on contiguous cells. Moreover, we have found that although exogenous AM cannot substitute for the AER in promoting the outgrowth of limb mesoderm, the AM antagonist AM(22-52) impairs the outgrowth and proliferation of limb mesoderm cultured in the presence of the AER. This is consistent with the possibility that inhibition of endogenous AM activity in the AER impairs the ability of the AER to promote the proliferation of limb mesoderm.

Materials and Methods

Immunohistochemistry

Immunohistochemical analysis of the distribution of AM immunoreactivity in serially sectioned chick limb buds that had been fixed in Bouin's was performed essentially as described by Gould *et al.* (1992) using a Vectastain Elite ABC kit (Vector) and a 1:500 dilution of a polyclonal antibody generated against a synthetic peptide encompassing the C-terminal 30 amino acids of human AM (PO72 in Martínez *et al.,* 1995). This antibody cross-reacts with AM from a variety of species, and its preparation, characterization, and specificity is described in detail in Martínez *et al.* (1995). In control experiments, we found that no immunostaining was

detectable with antiserum that had been preabsorbed to purified human AM coupled to Sepharose.

Preparation of Limb Buds from Limbless Mutant Chick Embryos

Windows were cut into eggs obtained from mating members of *limbless* heterozygous chickens maintained at the University of Connecticut, and the right forelimbs of embryos were removed and individually processed as described below. The windows in the shells of the donor embryos were sealed, and the donor embryos were reincubated until the phenotype of their remaining limb buds was clearly evident. Individual limb buds to be used for immunohistochemistry were fixed in Bouin's solution, processed to 70% ethanol, and stored in 70% ethanol until their phenotype was established.

Preparation and Analysis of Organ Cultures

The ectoderm was removed from the posterior 2/3 of stage 20/21 (Hamburger and Hamilton, 1951) wing buds as previously described (Dealy and Kosher, 1995), and the posterior mesoderm was cultured in the presence or absence of 10^{-5}-10^{-8} M of human adrenomedullin (Peninsula Laboratories; Phoenix Pharmaceuticals) on nutrient agar substrates (Kosher *et al.,* 1979) containing serum-free BGJ$_b$/F12 (7/3) medium supplemented with 0.1% bovine serum albumin (Dealy and Kosher, 1995). In other experiments, the posterior 2/3 of stage 20/21 wing buds were cultured with the AER and limb ectoderm intact in the presence or absence of 10^{-5}-10^{-6} M of the AM antagonist, AM(22-52) (Peninsula Laboratories).

The morphogenesis of living explants was evaluated daily with the dissecting microscope and living explants were photographed. Cell proliferation was examined as described by Dealy and Kosher (1995) by immunohistochemical analysis of the incorporation of 5-bromodeoxyuridine (BrdU) into nuclei engaged in DNA synthesis during a 1 hr labeling period with BrdU on days 3 and 4 of culture.

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