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Short Communication

Depressed adrenomedullin in the embryonic transforming growth factor-β1 null mouse becomes elevated postnatally

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ABSTRACT Transforming growth factor-beta (TGF-β) and adrenomedullin are multifunctional regulatory proteins which are expressed in developing embryonic and adult tissues. Because of their co-localization, TGF-β1 and adrenomedullin may be able to coordinately act to influence development and differentiation. In order to learn more about the biology of adrenomedullin in the absence of the effects of TGF-β1 *in vivo*, we examined adrenomedullin in the TGF-β1 null mouse. A generally lower amount of adrenomedullin was detected by immunohistochemical staining analysis in multiple tissues from embryonic TGF-β1 null mice compared to wildtype animals, including the heart, lung, brain, liver, and kidney, among others. In contrast, immunohistochemical staining for adrenomedullin was more intense in tissues of the postnatal TGF-β1 null mouse compared to the wildtype mouse. These observations were confirmed by quantitative real time RT-PCR for adrenomedullin in both embryos and postnatal animals, as well as in cultured mouse embryo fibroblasts from TGF-β1 null and wildtype mice. In addition, when cultured mouse embryo fibroblasts were treated with a neutralizing monoclonal antibody against TGF-β1, the levels of adrenomedullin expression were statistically reduced compared to untreated cells. Our data show that expression of adrenomedullin is reduced in tissues of the developing embryonic TGF-β1 null mouse compared to the wildtype mouse, but increases during postnatal development in TGF-β1 null mice. The elevated expression of adrenomedullin which occurs postnatally in the TGF-β1 null mouse may be a cause or a consequence of the multifocal wasting syndrome which is characteristic of postnatal TGF-β1 null mice.

KEY WORDS: *TGF-β, adrenomedullin, expression, development, mouse*

Several different polypeptide growth factors have been identified in normal developing mice, including adrenomedullin (AM) and transforming growth factor-beta (TGF-β). AM is a multifunctional regulatory molecule with several biological activities, and has been shown to stimulate or inhibit the proliferation of different cells (reviewed in López and Martínez, 2002). AM has been shown to be secreted by a variety of normal and malignant cells in culture (Martínez *et al.,* 1995; Miller *et al.,* 1996) and is expressed in a variety of embryonic and adult tissues (Montuenga *et al.,* 1997, 1998). Deletion of the AM gene results in embryonic lethality with hydrops fetalis, and cardiovascular abnormalities. TGF-β is also a multifunctional regulatory polypeptide that is the prototypical member of a large family of cytokines that controls many aspects of cellular function (reviewed in Massagué *et al.,* 2000). TGF-β1 is a potent immunoregulatory molecule that can both suppress and enhance cell functions *in vitro* and *in vivo*. Targeted disruption of gene expression through homologous recombination in mouse embryonic stem cells for TGF-β1 revealed a principal function in the maintenance of immunological homeostasis. Two to three weeks after birth, the TGF-β1 null mouse develops a wasting syndrome characterized by weight loss and multifocal infiltration of inflammatory cells in many organs such as the lungs, heart, and salivary glands, and progresses until the vital functions of the heart and lung are compromised (Schull *et al.,* 1992; Kulkarni *et al.,* 1993).

We have shown that expression of TGF-β1 and AM is regulated in a spatial and temporal manner such that overlapping patterns of TGF-β1 and AM expression occur in many tissues at the same stage of development and in the same cellular location in rodent embryogenesis (Montuenga *et al.,* 1998). The spatio-temporal

Abbreviations used in this paper: AM, adrenomedullin; MEF, mouse embryo-derived fibroblast; PBS, phosphate buffered saline; RT-PCR, reverse-transcription polymerase chain reaction; TGF-β, transforming growth factor-β; WT, wildtype.

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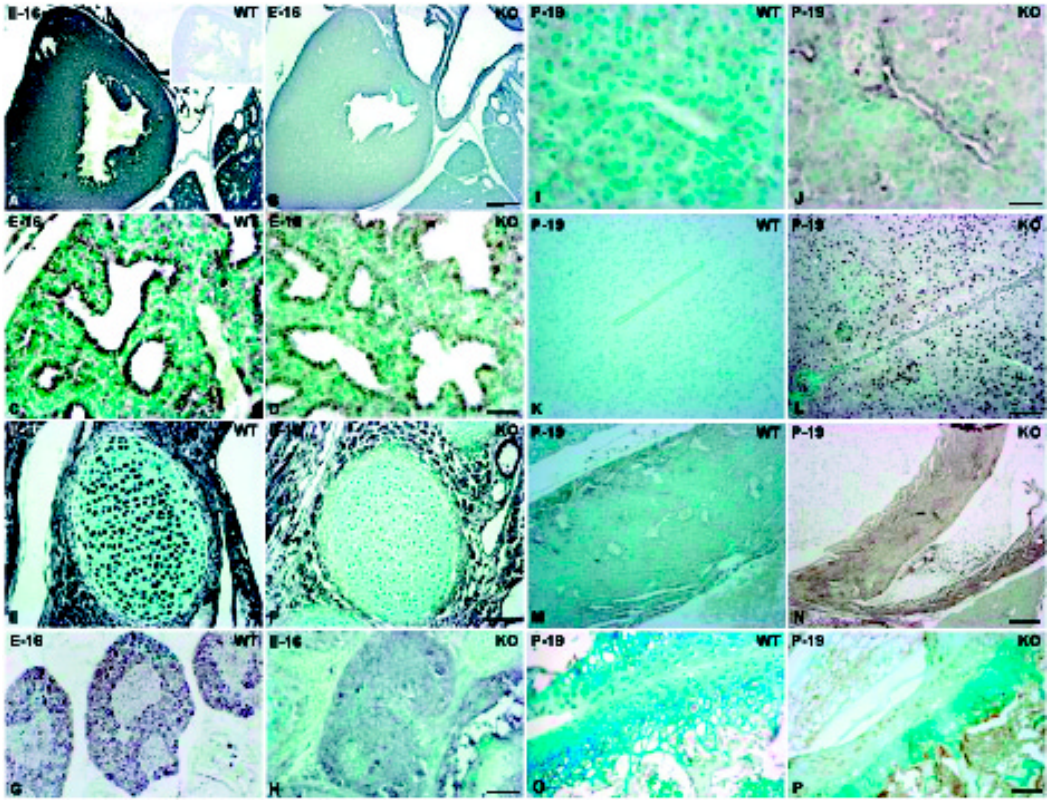


Fig. 1. Expression of AM protein in organs from developing TGF-β1 null and wildtype mouse embryos and postnatal mice. (A-H) *Immunohistochemical staining of E16 embryos.* **(I-P)** *Immunohistochemical staining of P-19 mice.* **(A,C,E,G)** *Wildtype (WT) embryos.* **(B,D,F,H)** *TGF-β1 null (KO) embryos.* **(I,K,M,O)** *Postnatal WT mice.* **(J,L,N,P)** *Postnatal TGF-β1 null mice. A,B, Heart and lung. C,D, Lung. E,F, Cartilage plate. G,H, Dorsal root ganglia. I,J, Liver. K,L, Brain. M,N, Heart. O,P, Cartilage and bone. Magnification: bars: A, B and A inset 250 μm; E, F, O, and P 100 μm; C, D, M, and N 50 μm; G, H-L 25 μm.*

patterns of expression of TGF-β1 and AM in cardiovascular, neural, and skeletal-forming tissues as well as in the main embryonic internal organs show striking similarities. Moreover, the lung, kidney, and intestine, in which epithelial-mesenchymal interactions occur, show similar patterns of TGF-β1 and AM expression. A more extensive knowledge of the biology of TGF-β could provide clues to the functions of AM. Here, we have examined AM during development of TGF-β1 null mice and compared it with wildtype (WT) littermates.

The immunohistochemical staining pattern of AM in TGF-β1 null mice was established and compared with WT littermates in several embryonic tissues ranging in age from E11 to E19. Figure 1A shows a low power image of the heart and lung of an E16 WT mouse in which both the ventricle and atrium as well as lung parenchyma show positive immunostaining for AM. In contrast, Fig. 1B shows weak immunostaining for AM in the heart and lung parenchyma of an E16 TGF-β1 null mouse. Immunohistochemical staining for AM was completely blocked when the AM antibody was preincubated with its peptide immunogen (Fig. 1A inset). Figure 1C shows a higher magnification of the lung parenchyma of an E16 WT mouse immunostained for AM, and compares it with the lung of a TGF-β1 null mouse of the same age (Fig. 1D). The intensity of immunostaining for AM in epithelial cells of the forming airways and in several isolated cells in the differentiating mesenchyme of the WT lung is considerably stronger than in the lung of the TGF-β1 null mouse. Figure 1E shows that while there is intense staining for AM in the cartilage plate of a developing rib of an E16 WT embryo, the intensity of staining for AM is markedly reduced in the chondrocytes in a TGF-β1 null mouse of the same age (Fig. 1F). The immunostaining pattern of AM in the dorsal root ganglia of an E16 WT mouse (Fig. 1G) shows moderately intense staining of neurons while these same neurons show only weak immunostaining in the TGF-β1 null mouse embryo (Fig. 1H). Table 1 shows a comparison of immunohistochemical staining intensity for AM in several tissues from E16 and E20 TGF-β1 null and WT mice. The intensity of immunohistochemical staining for AM was strong in heart and dorsal root ganglia and moderate in lung, liver, kidney, chondrocytes, and brain of embryonic WT mice of both ages compared to TGF-β1 null mice. Our demonstration of reduced levels of AM protein in several tissues of the embryonic TGF-β1 null mouse compared to its wildtype littermates shows that AM expression is affected by deletion of the TGF-β1 gene. Whether reduced expression of AM in the embryonic TGF-β1 null mouse is a direct effect of deletion of the two TGF-β1 alleles or a secondary effect that follows from intermediate changes is not known.

To investigate the localization of AM protein in postnatal development of TGF-β1 null mice, the immunohistochemical staining pattern of AM was examined in several organs of postna-

TABLE 1

IMMUNOHISTOCHEMICAL STAINING INTENSITY OF AM PROTEIN DURING MOUSE EMBRYOGENESIS AND POSTNATAL DEVELOPMENT IN TGF-β1 NULL AND WILDTYPE MICE

	E16		E20		P-7		P-17		P-19	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Heart	3	1	3	1	3	1	2	2	1	2
Lung	2	1	2	1	2	1	1	2	1	2
Liver	2	1	2	1	2	1	1	2	1	2
Kidney	2	1	2	1	2	1	1	2	1	2
DR Ganglia	3	1	3	1	3	1	2	2	1	2
Chondrocytes	2	1	2	1	2	1	1	2	1	2
Brain	2	1	2	1	2	1	1	3	1	3

Staining intensity is ranked 0, no; 1, weak; 2, moderate; 3, intense. KO, TGF-β1 null; WT, wildtype.

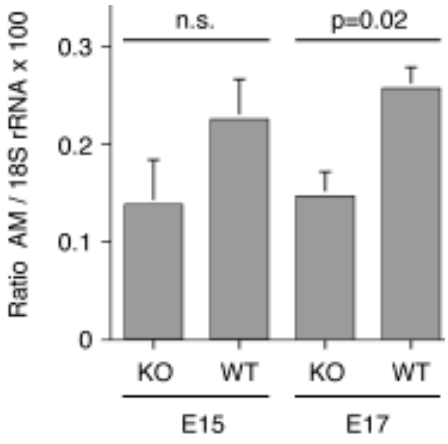
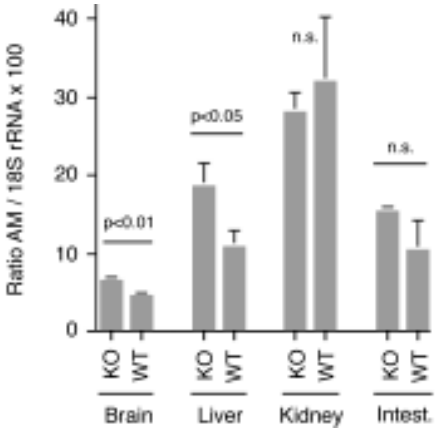


Fig. 2. Quantitative real time RT-PCR for *adrenomedullin* mRNA in whole embryo extracts from TGF-β1 null and wildtype animals at E15 and E17 of intrauterine development. *The statistical significance of the differences observed between TGF-β1 null (KO) and wildtype (WT) animals are expressed as a "p" value or as not significant (n.s.).*

tal TGF-β1 null mice and compared with wildtype littermates in several tissues ranging in age from P-7 to P-19. While Fig. 1I shows weak immunostaining for AM in the liver parenchyma of a P-19 WT mouse, the hepatocytes, and in particular, the epithelial cells of the bile duct in the liver of the TGF-β1 null mouse show more intense staining for AM (Fig. 1J). Figure 1K shows that while neurons in the brain of the WT mouse have a pattern of general weak staining, many of the neurons in the brain of the TGF-β1 null mouse have strong immunostaining (Fig. 1L). In the developing postnatal heart, although some myocytes show some immunostaining for AM, especially in the periphery of the heart of the WT mouse (Fig. 1M), the intensity and distribution of AM immunostaining is considerably stronger and more pronounced in the cardiac myocytes of the TGF-β1 null mouse (Fig. 1N). Cartilage in the area of endochondral ossification as well as in forming bone cells are more strongly immunostained for AM in the P-19 TGF-β1 null mouse (Fig. 1P) than in the WT littermate (Fig. 1 O). We also performed immunohistochemical analysis for AM on several organs from postnatal TGF-β1 null and WT mice over a range of ages, including P-7, P-14, and P-19 mice (Table 1). The intensity of immunohistochemical staining for AM was either intense or moderate in all tissues examined of the P-7 WT mice compared to weak staining in TGF-β1 null mice of the same age. The intensity of staining for AM decreased to either moderate or weak in all of the tissues examined in P-17 WT mice, while it increased to moderate to strong levels in P-17 TGF-β1 null mice. Table 1 also shows that immunostaining continued to decrease in the heart and dorsal root ganglia and moderately in lung, liver, kidney, chondrocytes, and brain of embryonic WT mice of both ages compared to TGF-β1 null mice. A gradual elevation in AM from younger to older postnatal TGF-β1 null mice would suggest that this is a cumulative effect and may contribute to the lethality. The coordinated change in AM suggests that it may play a key role in the inflammatory response as well. AM appears to play a pivotal role in both reprioritizing the biological needs of tissues and organs during the various phases of inflammatory response as well as a role in restoring homeostatic equilibrium to the organism. Increased AM expression in the postnatal TGF-β1 null mouse may mediate either pro-inflammatory activities or oppose these actions. However, if AM opposes inflammatory activities in the postnatal TGF-β1 mouse, it is unable to prevent inflammation and death in this mouse.

To confirm the observations made by immunohistochemcal analysis, namely that AM levels are higher in wildtype fetal mice than in their TGF-β1 null littermates, total RNA was extracted from whole embryos of different ages, cDNA was synthesized, and real time polymerase chain reaction (PCR) amplification was performed with primers for AM, and also for 18S rRNA as a house-keeping gene.



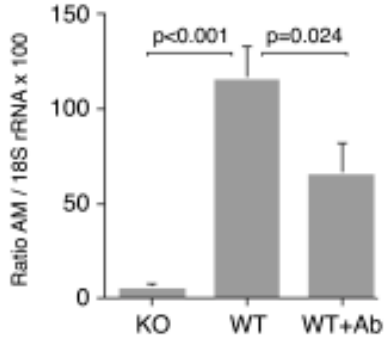


Fig. 3 (Left). Quantitative real time RT-PCR for *adrenomedullin* mRNA in tissue extracts from TGF-β1 null and wildtype animals at P14 of postnatal development. *The statistical significance of the differences observed between TGF-β1 null (KO) and wildtype (WT) animals are expressed as a "p" value or as not significant (n.s.).*

Fig. 4 (Right). Quantitative real time RT-PCR for *adrenomedullin* mRNA in primary cultures of embryonic fibroblasts obtained from TGF-β1null and wildtype mice. *Wildtype fibroblasts were exposed to a neutralizing antibody against TGF-β1 (WT+Ab) or to saline (WT), and TGF-β1 null (KO) fibroblasts were similarly exposed to saline before RNA extraction. The statistical significance of the differences observed between groups is expressed as a "p" value.*

Figure 2 shows that the expression levels for AM mRNA, corrected by house-keeping gene contents, were higher in the WT animals than in their TGF-β1 null littermates at E15 and E17. At day 15, the difference was not statistically significant, but at day 17, the p value was 0.02.

Real time RT-PCR amplification was also used to evaluate the expression of AM mRNA during postnatal development in the TGF-β1 null mouse. Figure 3 shows the results obtained for P-14 mice. A statistically significant decrease in AM mRNA expression was found in the brain and liver of wildtype animals when compared to their TGF-β1 null littermates. Differences of expression in other organs, such as the kidney and the intestine, did not reach statistical significance, but this is clearly understandable when we consider that for molecular techniques, a complete homogenate of the tissue has to be prepared, and in some cases a dilution effect may obscure the differences observed by more cell-type specific *in situ* techniques such as immunohistochemistry.

To complement our *in vivo* experiments, total RNA was also extracted from primary cultures of mouse embryonic fibroblasts (MEF) obtained from E18 TGF-β1 null and WT mice, cDNA was synthesized, and real time PCR amplification was performed with primers for AM as *in vivo*. Figure 4 shows a statistically significant higher level of AM mRNA in cultured MEFs from wildtype mice compared to TGF-β1 null mice and parallels our *in vivo* results. There was also a decrease in the level of AM mRNA expression in MEFs from wildtype mice after treatment with a neutralizing antibody to TGF-β1 with a p value of 0.024, indicating that neutralization of TGF-β1 induces downregulation of AM levels, in agreement with our observations in TGF-β1 null animals. These studies indicate that TGF-β1 regulates AM expression. One mechanism by which TGF-β1 may regulate AM mRNA expression may be at the level of the AM promoter. Analysis of the sequence of the mouse AM promoter shows at least one potential Smad binding element by which TGF-β1 signaling may be able to regulate AM mRNA expression, and this will be needed to be addressed in future studies.

At this time, although we have demonstrated that an increase in AM occurs in several tissues of the postnatal TGF-β1 null mouse, we are unable to determine whether this increase is a cause or a consequence of the pathology. It is also unclear whether increases in AM in specific cells of affected tissues reflect an overflow from local sites of production and action, or whether in some conditions, increased AM has a hormonal function causing a decrease in vascular resistance and a decrease in blood pressure, among other effects. Our findings show an interaction between TGF-β1 and AM and raise additional questions about their functions in the diseased state.

Experimental Procedures

TGF-β1 null and WT mice were sacrificed using carbon dioxide inhalation, embryos were removed from the uterus, and embryos and postnatal tissues were placed immediately into formalin for paraffin embedding and sectioning. Immunohistochemical staining for AM was performed using the avidin-biotin complex technique with a previously characterized polyclonal antibody to AM (Miller *et al.,* 1996). In addition, MEFs harboring the null or wildtype TGF-β1 allele were derived from E18 mice to establish TGF-β1 null and WT fibroblasts that were cultured over multiple passages to obtain sufficient primary cells. The MEFs from wildtype mice were treated for 24 h with 30 μg/ml neutralizing monoclonal TGF-β1 antibodies from R&D Systems (Minneapolis, MN).

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