J. Anat. (1999) **195**, pp. 75–86, with 4 figures Printed in the United Kingdom

Angiotensin II is a growth factor in the peri-implantation rat embryo

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(*Accepted 16 March 1999*)

ABSTRACT

Angiotensin II (ANG II) is increasingly recognised as a growth factor, both in its own right and through interactions with other growth factors. There is a high density of ANG II receptors in the rat fetus, especially the AT2 receptor, the function of which is still uncertain. We have now studied the effects of ANG II on growth and development in the rat embryo in vitro between d 9.5 and 11.5, and characterised the receptor subtype mediating these effects. Embryos were cultured in whole rat serum, a high molecular weight retenate after ultrafiltration of whole rat serum, retenate with angiotensin II and retenate with ANG II and AT1 or AT2 receptor blockers. Growth and development were scored using conventional methods. Culture in retenate was associated with a marked reduction in growth and development by comparison with whole rat serum. This was partly, and significantly ($P < 0.001$), reversed by angiotensin II. The optimum concentration of angiotensin II was found to be angiotensin II 10^{-11} M, within the physiological range. Angiotensin II had highly significant effects on both somatic ($P < 0.001$) and yolk sac/allantoic ($P < 0.005$) development. The latter effects suggest a role for angiotensin II in placentation. The effects of angiotensin II were blocked by PD123319, an AT2 blocker, but not by GR117289, an AT1 blocker. Interestingly, culture in retenate with GR117289 without added angiotensin II was also associated with some increase in growth ($P < 0.05$). Angiotensin II in low concentrations was measurable in the retenate, presumably arising from the action of endogenous renin on angiotensinogen. We therefore postulate that this effect of GR117289 was due to the action of endogenous angiotensin II on 'uncovered' AT2 receptors. This study has thus demonstrated a direct growth promoting effect of angiotensin II during organogenesis in the whole rat embryo in vitro. This effect is mediated through the AT2 receptors.

Key words: Renin-angiotensin receptors; angiotensin AT1 and AT2 receptors; pregnancy.

INTRODUCTION

Angiotensin II (ANG II) is a major component of the renin-angiotensin system (RAS) and has long been accepted as an important regulator of blood pressure and fluid and electrolyte balance. An increasing body of evidence has demonstrated the involvement of ANG II in the control of cell growth (Schelling et al. 1991). ANG II stimulates proliferation in several cell types in vitro and in vivo, for example in bovine adrenal glomerulosa cells (Tian et al. 1995), rat vascular smooth muscle cells (Dudley et al. 1991) and ventral spinal cord (Iwasaki et al. 1991) and chick chorioallantoic vascular arcade development (le Noble et al. 1996). Additionally ANG II induces the expression of immediate-early growth response genes such as *c-myc* and *c-fos* (Riordan, 1995) and activates certain protein kinases known to be important in the regulation of the cell cycle (Dzau, 1994). To mediate its effects ANG II must interact with specific receptors. The majority of the known physiological effects of ANG II, including growth promotion, are mediated by the ANG II type 1 (AT1) receptor. This receptor is expressed in the adult in classical ANG II target

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tissues such as the kidney, heart, adrenal gland and large blood vessels. ANG II also interacts with the ANG II type 2 (AT2) receptor which may play a role in growth inhibition (Stoll et al. 1995; Tsuzki et al. 1996) and apoptosis (Yamada et al. 1996). The AT2 receptor also appears to be coupled to various phosphatases, including mitogen-activated protein kinase phosphatase 1, and can inhibit AT1 receptor-mediated effects (see Unger et al. 1996). The balance of receptor density between the 2 subtypes may thus be significant in the regulation of cellular proliferation and growth.

During the period of organogenesis there is extensive cellular proliferation, differentiation and apoptosis. Many growth factors such as epidermal growth factor (Lee & Han, 1990) and vascular endothelial growth factor (see Breier et al. 1997) have been demonstrated to regulate normal embryonic development. Hormones such as ANG II may also regulate embryonic development but this has yet to be tested experimentally. ANG II binding sites have been found on fetal rat cell membranes by at least embryonic d 10, increasing in density during gestation such that on d 15 the concentration of binding sites equals that in the adult adrenal (Jones et al. 1989). During gestation binding sites were readily detectable in classic ANG II target tissues such as the adrenal glands and blood vessels but were also observed in skin, mesenchymal tissue and connective tissue; areas not normally shown to contain ANG II binding sites in the adult (Millan et al. 1989). In these novel areas binding was shown rapidly to decrease within 1 d of birth (Millan et al. 1989). Subsequent investigation using receptor subtype specific antagonists has demonstrated that the majority of fetal ANG II binding sites are AT2 receptors (Grady et al. 1991; Tsutsumi et al. 1991; Feuillan et al. 1993) which are expressed transiently in the skin, skeletal muscle and in undifferentiated mesenchyme during gestation (Grady et al. 1991). Such abundant but transient expression of the AT2 receptor has led to the hypothesis that ANG II may regulate growth during embryonic and fetal development.

The present study has investigated the role of ANG II during organogenesis in the whole rat embryo using in vitro culture techniques. Initially ANG II was added to whole rat serum depleted of low molecular weight molecules by prolonged filtration (retenate) to determine the growth promoting effects of ANG II during organogenesis. The roles of the AT1 and AT2 receptor subtypes were then investigated in normal development and during the growth promoting effects of ANG II in retenate culture.

METHODS AND MATERIALS

Whole embryo culture

Female Wistar rats were mated overnight and pregnancy timed from midnight preceding the morning of discovery of a copulation plug. Pregnant rats were killed on d 9.5 by ether overdose and the embryos explanted according to a standard method of embryo culture (New, 1978). Briefly, explanted embryos were cultured in glass culture bottles containing homologous, heat-inactivated rat serum in a roller incubator at 37 °C. Embryos were gassed immediately following explantation with a mixture of 5% O_2, 5% CO_2 and 90% N_2, and after a further 24 h with 20% O_2, 5% CO_2 and 75% N_2 and 4 h before harvesting with 40% O_2, 5% CO_2 and 55% N_2. On d 11.5 embryos were removed from culture and assessed morphologically by one observer (CT), blinded to treatment group, according to the scoring system of Van Maele Fabry et al. (1990). For each embryo a total morphological score was allocated based on the development of various primordia (using a 5-point scale for each), and on the mean yolk sac diameter, the crown–rump length and somite number. The primoridia studied included neural tube, otic, optic, maxillary, mandibular, branchial bar, yolk sac circulatory system and allantoic development. Embryonic and yolk sac protein contents were determined by biochemical assay (Lowry et al. 1951).

Serum filtration

Filtration of rat serum was carried out using a Macrosep centrifugal concentrator with a nominal molecular weight exclusion point of 30 kDa. 20 ml heat inactivated homologous rat serum was pooled, from which 5 ml was retained as control serum. To each centrifugal concentrator 15 ml serum was added and then centrifuged for a total of 8 h at 3500 rpm (1000 ***g***) at 4 °C. After 2 h the low molecular weight filtrate was removed and the high molecular weight fraction was diluted to its original volume with distilled water. This procedure was repeated after 4 and 6 h to prevent the retenate becoming too viscous to be efficiently filtered. At the end of filtration the high molecular weight fraction was reconstituted to 15 ml with double strength Hank's balanced salt solution (600 mOsm) and the final osmolarity and pH corrected to 290 to 330 mOsm and 7.8 respectively. Prior to use in culture retenate was sterile filtered by passage through a Minisart NML 0.2 mm filter unit (Sartorius, Göttingen, Germany) and supplemented with 2 mg/ml glucose and 10 µl/ml minimal essential medium (MEM) vitamin solution (Sigma, Poole, UK).

Experiments undertaken

To determine the growth promoting effects of ANG II during development and the optimum concentration, human ANG II (Sigma, Poole, UK) was added to retenate culture at concentrations of 10^{-5}, 10^{-8}, 10^{-11} and 10^{-14} M. Culture in retenate and whole rat serum were included in each experiment as controls.

The effect of inhibiting the activity of the AT1 and AT2 receptor subtypes during organogenesis was investigated using the specific receptor subtypes PD123319 (Parke Davis, Eastleigh, UK), to inhibit the activity of the AT2 receptor and GR117289 (Glaxo Wellcome Research and Development, Stevenage, UK), an AT1 receptor antagonist. Initially embryos were cultured in whole rat serum in the presence of 10^{-6} M GR117289 and PD123319. To investigate the role of the AT1 and AT2 receptors in the ANG II-induced increase in embryonic development embryos were cultured in retenate in the presence of ANG II (10^{-11} M), or the specific antagonist or in the presence of both ANG II and the antagonist. Concentrations of GR117289, PD123319 were added to retenate culture at a concentration of 10^{-6} M.

The concentrations of endogenous ANG II in whole rat serum and retenate were measured by radioimmunoassay following purification by HPLC before and after culture (Dr P. J. J. Admiraal, Rotterdam, The Netherlands).

Statistical analysis

Results are given as mean and standard errors where data are normally distributed and as median values with their associated interquartiles otherwise. The effect of culture in retenate with and without added ANG II at 10^{-11} M (see Results) was analysed over the entire series of experiments using Student's t test or the Mann-Whitney U test as appropriate. Subsequent analyses of the effects of each receptor blocker were conducted using conventional 1-way ANOVA followed by Duncan's multiple range test or the Kruskal Wallis distribution-free ANOVA as appropriate. Statistical significance of differences between groups was assumed at a P value of < 0.05.

RESULTS

Culture of embryos in the presence of ANG II

Overall, embryos cultured in retenate (n = 57) were significantly ($P < 0.001$) growth retarded when compared with those cultured in whole rat serum (n = 52). Morphologically embryos had closed neural tubes, only one branchial bar, retarded otic and optic development and shortened tails. The addition of ANG II to retenate improved embryonic development (Fig. 1) in a dose-dependent manner, the maximum increase in development being observed at 10^{-11} M. A total of 63 embryos were cultured in retenate + ANG II (10^{-11} M) during the entire series of experiments. In the presence of 10^{-11} M ANG II median morphological score was increased from 34.0 (29.8, 37.3) in retenate alone to 40.0 (35.0,45.0; $P < 0.001$) and somite number was increased from 10.2 ± 0.4 to 12.9 ± 0.5 ($P < 0.001$). Morphologically these embryos had closed neural tubes, improved optic and otic development ($P < 0.001$ for all), an increase in the number of branchial bars ($P < 0.002$) and improved mandibular development ($P < 0.001$). In addition embryos had significantly greater crown rump lengths (2.71 ± 0.05 mm; $P < 0.001$) than retenate controls (2.41 ± 0.06 mm). The embryo protein content rose from 80.9 ± 3.5 μg to 90.6 ± 3.3 μg ($P < 0.05$). Scores for both the yolk sac circulatory system and allantoic development were increased following culture in retenate with ANG II at 10^{-11} M ($P < 0.005$; $P < 0.001$) as was yolk sac diameter (3.01 ± 0.04 compared with 2.84 ± 0.05 mm; $P < 0.01$).

A decrease in the effect of ANG II was observed as embryos were exposed to concentrations greater than 10^{-11} M. No significant growth promoting effects were observed in the presence of 10^{-8} M ANG II and at the highest concentration used (10^{-5} M) embryos had a significantly reduced protein content (40.6 ± 8.8 μg compared with controls (72.3 ± 5.5 μg).

Exposure of preimplantation rat embryos to ANG II receptor antagonists

Embryos were exposed to the AT2 antagonist PD123319 and the AT1 antagonist GR117289 during culture in whole rat serum. No significant differences were observed in the development of embryos cultured in the presence of the above antagonists when compared with whole rat serum controls (data not shown).

Exposure of embryos to ANG II receptor antagonists in retenate culture in the presence of ANG II

PD123319. Embryos cultured in the presence of retenate with PD123319 alone (n = 21) were not significantly different in development from retenate controls. Addition of the antagonist in the presence of ANG II 10^{-11} M (n = 21) abolished the ANG II-induced increase in embryonic development (Fig. 2). Exposure of embryos to PD123319 in the presence of

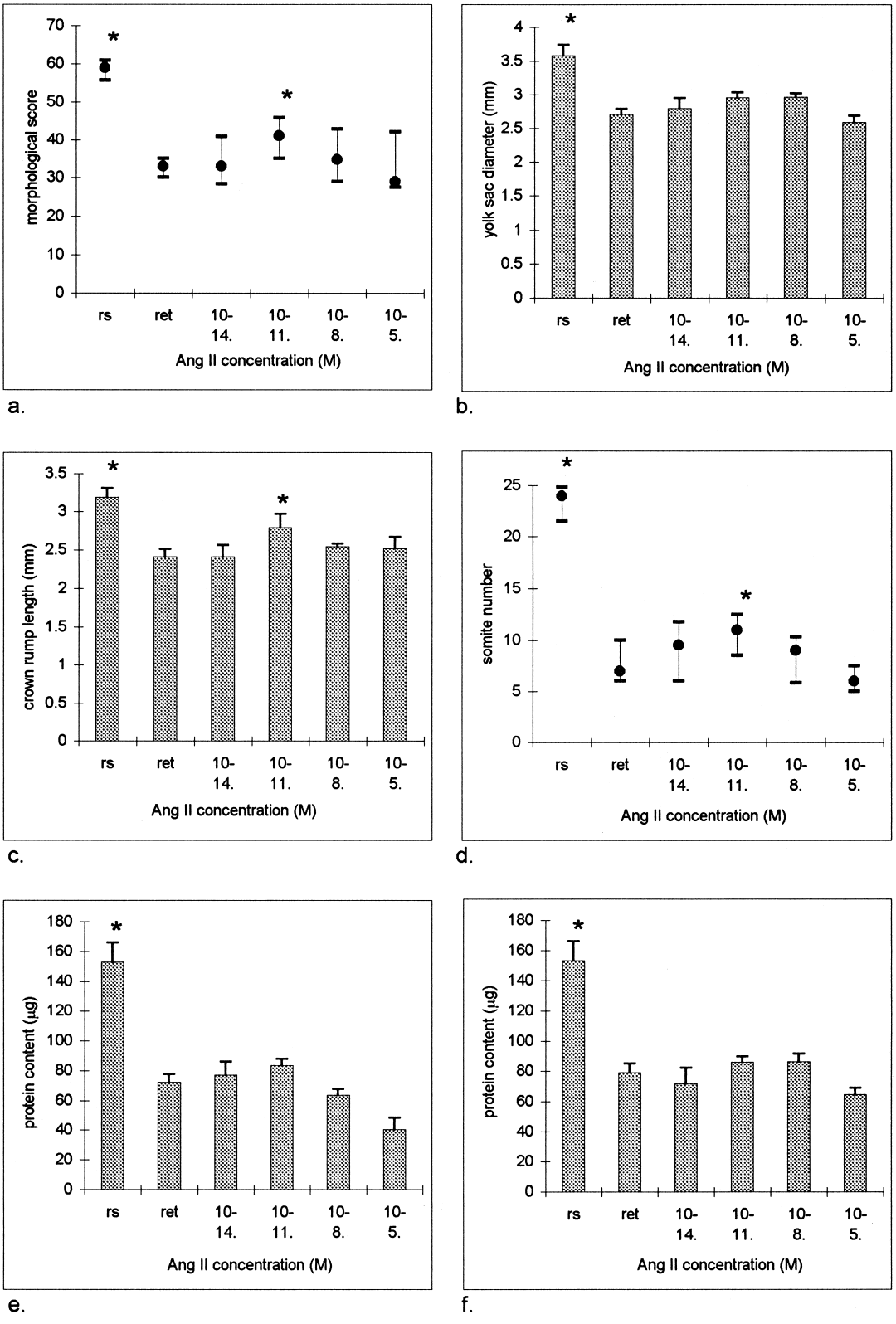

Fig. 1. The growth promoting effect of ANG II during early organogenesis of the rat embryo in retenate culture. (*a*) Morphological score; (*b*) yolk sac diameter; (*c*) crown-rump length; (*d*) somite number; (*e*) embryonic protein content; (f) yolk sac protein content. rs, whole rat serum; ret, retenate. In panels *a* and *d*, results are median and interquartile range for at least 10 embryos. In panels *b*,*c*,*e* and *f*, results are mean $\pm$ S.E.M. *Significant difference in growth from retenate control, $P < 0.05$.

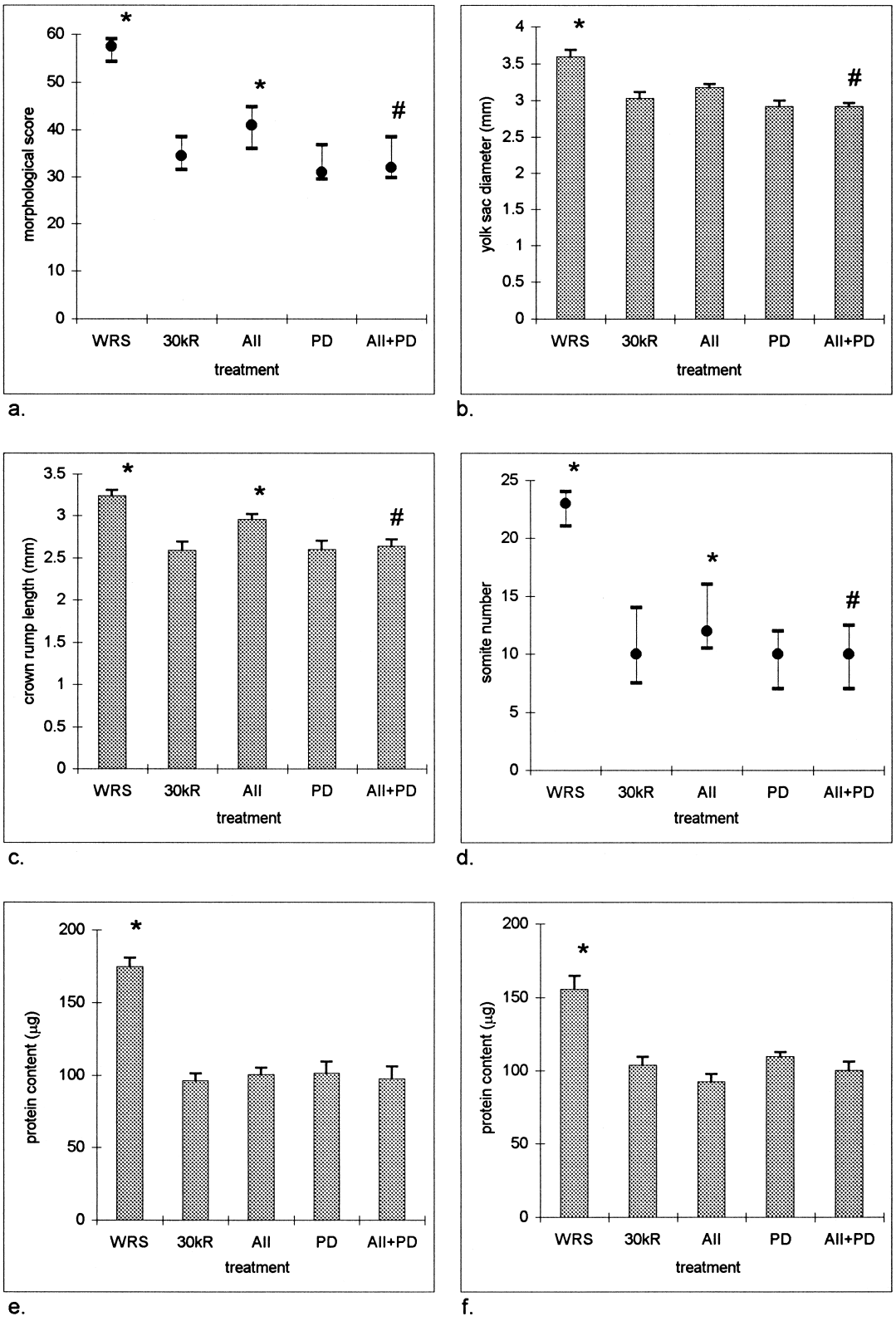

Fig. 2. Effect of the addition of the AT2 receptor antagonist PD123319 during culture of d 9.5 rat embryos in ANG II-supplemented (10^{-11} M) retenate. (*a*) Morphological score; (*b*) yolk sac diameter;(*c*) crown-rump length; (*d*) somite number; (*e*) embryonic protein content; (*f*) yolk sac protein content. WRS, whole rat serum; 30 kR, retenate; AII, angiotensin II (10^{-11} M); PD, PD123319 (10^{-6} M); AII + PD, angiotensin II (10^{-11} M) and PD123319 (10^{-6} M). In panels *a* and *d*, results are median and interquartile range for at least 10 embryos. In panels *b*, *c*, *e* and *f*, results are mean ± S.E.M. *Significant difference in growth from retenate control; # significant difference in growth from embryos cultured in ANG II-supplemented retenate, $P < 0.05$.

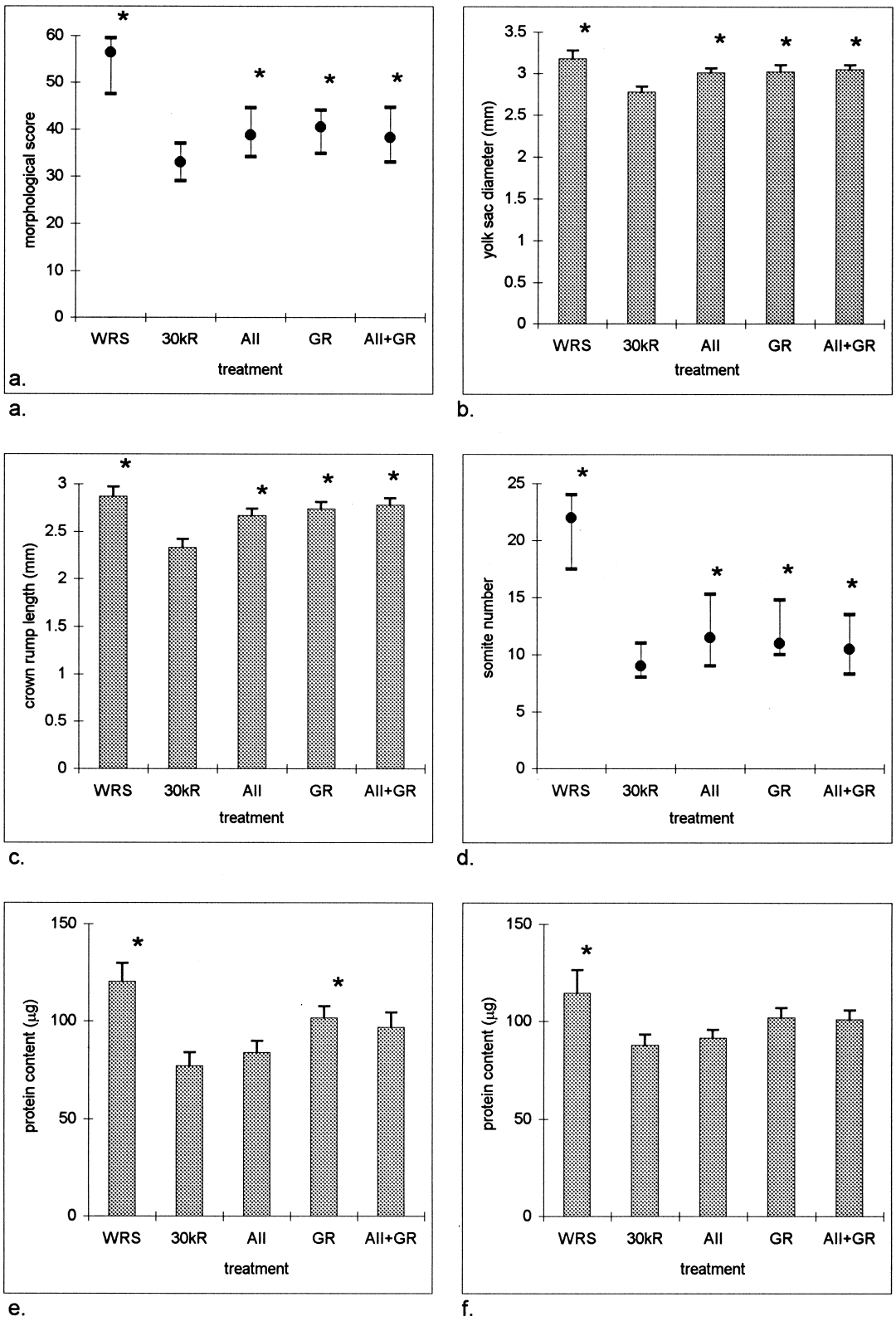

Fig. 3. Effect of the addition of the AT1 receptor antagonist GR117289 during culture of d 9.5 rat embryos in ANG II-supplemented retenate. (*a*) Morphological score; (*b*) yolk sac diameter;(*c*) crown-rump length; (*d*) somite number; (*e*) embryonic protein content; (*f*) yolk sac protein content. WRS, whole rat serum; 30 kR, retenate; AII, angiotensin II (10^{-11} M); GR, GR117289 (10^{-6} M); AII + GR, angiotensin II (10^{-11} M) and GR117289 (10^{-6} M). In panels *a* and *d*, results are median and interquartile range for at least 10 embryos. In panels *b*, *c*, *e* and *f*, results are mean ± S.E.M. *Significant difference in growth from retenate control.

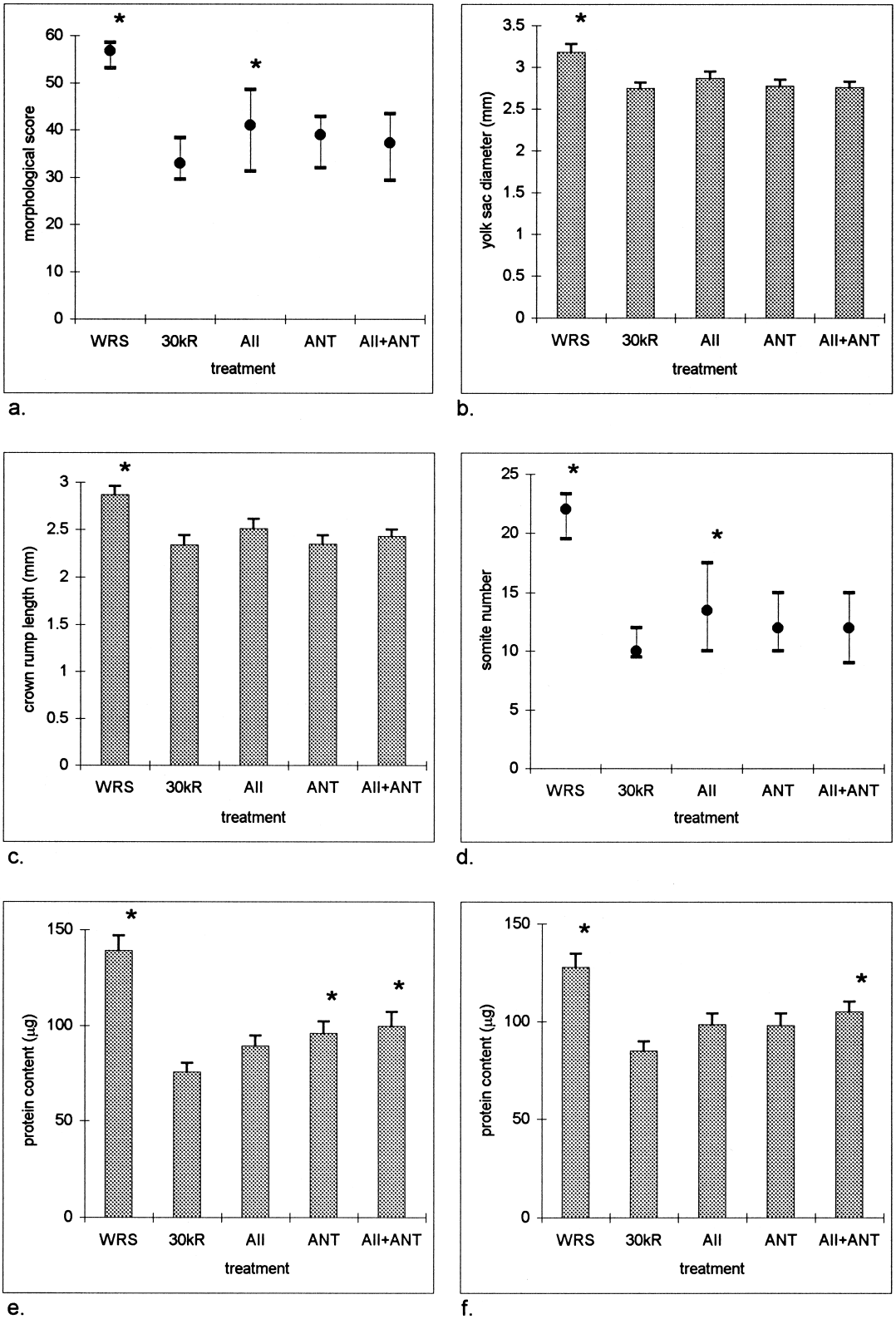

Fig. 4. Effect of the addition of AT1 and AT2 receptor antagonists during culture of d 9.5 rat embryos in ANG II-supplemented retenate. (*a*) Morphological score; (*b*) yolk sac diameter; (*c*) crown-rump length; (*d*) somite number; (*e*) embryonic protein content; (*f*) yolk sac protein content. WRS, whole rat serum; 30kR, retenate; AII, angiotensin II (10^{-11} M); ANT, GR117289 (10^{-6} M) and PD123319 (10^{-6} M); AII + ANT, angiotensin II (10^{-11} M) and GR117289 and PD123319 (10^{-6} M). In panels *a* and *d*, results are median and interquartile range for at least 10 embryos. In panels *b*, *c*, *e* and *f*, results are mean ± S.E.M. *Significant difference in growth from retenate control.

ANG II resulted in a significant ($P < 0.001$) decrease in morphological score from 41.0 (36.0, 44.8) in the presence of 10^{-11} M ANG II alone to 31.0 (29.5, 36.8). Crown-rump length was reduced (2.64 ± 0.08 mm vs 2.96 ± 0.06 mm; $P < 0.02$) under these conditions as was somite number (9.7 ± 0.6 vs 12.7 ± 0.7; $P < 0.01$). The addition of PD123319 to retenate + ANG II completely abolished the stimulatory effects of ANG II alone on allantoic ($P < 0.01$) and yolk sac ($P < 0.001$) development and yolk sac diameter (2.92 ± 0.05 vs 3.18 ± 0.05 mm; $P < 0.005$). PD123319 also abolished the ANG II-induced increase in otic and optic development, mandibular and forelimb bud development ($P < 0.005$ for all) and the number of branchial bars ($P < 0.02$).

GR117289. Addition of GR117289 to retenate culture without added ANG II (n = 20) resulted in improved embryonic development; significant increases ($P < 0.05$) were observed in morphological score (40.5 (34.4, 44.0) vs 33.0 (29.0, 37.0), yolk sac diameter (3.02 ± 0.08 mm vs 2.78 ± 0.06 mm), crown rump length (2.74 ± 0.07 mm vs 2.33 ± 0.09 mm), somite number (12.2 ± 0.8 vs 8.9 ± 0.5) and embryonic protein content (101.7 ± 5.9 μg vs 77.2 ± 6.7 μg; Fig. 3). In the presence of both ANG II and GR117289 (n = 20) median morphological score was significantly increased ($P < 0.05$) from 33.0 (29.0, 37.0) in retenate alone to 36.5 (33.0, 44.6), yolk sac diameter was increased from 2.78 ± 0.06 mm to 3.05 ± 0.05 mm. Statistically significant increases in crown-rump length (2.78 ± 0.07 mm vs 2.33 ± 0.09 mm) and somite number (11.5 ± 0.9 vs 8.9 ± 0.5) were also observed.

PD123319 and GR117289. To determine the effects of inhibiting the AT1 and AT2 receptors simultaneously embryos were exposed to PD123319 and GR117289 in ANG II-supplemented retenate culture (Fig. 4). In the presence of PD123319 and GR117289 alone (n = 25) a significant increase in embryonic protein content was observed (95.9 ± 6.3 μg vs 75.5 ± 4.8 μg; $P < 0.05$) although no other parameters were increased. Similar increases in embryonic protein (99.6 ± 7.5 μg vs 75.5 ± 4.8 μg; $P < 0.01$) and yolk sac protein (105.1 ± 5.2 μg vs 75.5 ± 4.8 μg; $P < 0.01$) were observed in the presence of ANG II and the 2 antagonists (n = 28).

DISCUSSION

Previously published work (Tebbs et al. 1997) using this culture system has shown that essential growth-promoting factors are removed from rat serum during filtration, but that the process of filtration itself is not detrimental to embryonic growth. Many growth factors are of low molecular weight, and will therefore be removed during filtration. Their replacement allows the specific effects of individual growth factors to be assessed, although interactions between such factors may be lost.

ANG II is a well characterised vasoconstrictor important in cardiovascular homeostasis. However, evidence has accumulated in support of a role for ANG II in growth promotion (Schelling et al. 1991). This study supports our hypothesis that ANG II may be able to act as a growth regulating factor during early organogenesis in the rat embryo. Initially embryos were cultured in serum depleted of low molecular weight molecules by prolonged filtration which resulted in growth retardation when compared with those cultured in whole serum. Filtration will remove basal ANG II (MW 1.036 kDa) but not angiotensinogen, renin or angiotensin converting enzyme (ACE)(MW: 56–60 kDa, 58 kDa and 140–170 kDa respectively). ACE is known to be present in plasma as well as in pulmonary and other vascular endothelia (Peach, 1977). ANG II could therefore have been being generated during incubation. Preliminary measurements (see Table) showed that low levels of ANG II were indeed detectable in retenate. Decreases in ANG II concentration were observed following 48 h culture in both whole rat serum and retenate.

The addition of exogenous ANG II to retenate culture significantly improved embryonic development with a maximal effect at 10^{-11} M. This concentration is in the physiological range of ANG II; circulating concentrations of ANG II in the rat are estimated to be 10^{-11} M to 10^{-10} M (Baylis et al. 1997) and tissue ANG II concentrations are extremely low (De Silva et al. 1988). The addition of high concentrations of ANG II (10^{-8} M to 10^{-5} M) to retenate resulted in a decrease in the growth promoting effect of ANG II. This may be due to the phenomenon of down regulation. Exposure to high concentrations of a growth factor results in internalisation of the growth factor/receptor complex, and consequently the reduction in the cell's responsiveness to the factor (Raff, 1976). Downregulation of AT1 receptors has been documented in rat vascular smooth muscle and mesangial cells where concentrations in excess of 10^{-8} M ANG II caused a decrease in receptor expression (Makita et al. 1992; Lassegue et al. 1995). The expression of AT2 receptors is also regulated by growth related factors (Lassegue et al. 1994). In rat ovarian granulosa cells which express only AT2 sites,

Table. *Endogenous ANG II concentrations measured in whole rat serum and in retenate at the start of culture and at the time of harvesting*

Culture conditions	ANG II concentration before culture (pmol/l)	ANG II concentration after culture (pmol/l)
Whole rat serum	14.7	4.1
Retenate	12.6	7.5

ANG II binding was decreased in the presence of ANG II, so down regulating its own binding site (Pucell et al. 1988).

The growth promoting effects of ANG II in retenate culture may be due to a compensatory effect of the molecule for those lost nonspecifically during the filtration procedure. With respect to this, culture of rat embryos in a similar depleted serum system supplemented with insulin suggested that embryos are capable of utilising exogenous insulin (Pratten et al. 1988), an observation confirmed by specific removal of insulin by affinity chromatography (Travers et al. 1989). Rat embryos utilise ANG II during normal culture in rat serum; the concentration of ANG II in whole serum dramatically decreases following 48 h culture in vitro (Table). Additionally ANG II is utilised by embryos cultured in retenate culture. In vivo embryos may use ANG II from 2 different sources; the embryo itself and the maternal uterus. Angiotensinogen is expressed by the rat embryo from at least d 11 and increases to reach a plateau on d 17 (Lee et al. 1987). Additionally, renin (Richoux et al. 1987) and ACE (Jung et al. 1993) are expressed during fetal life and may form a functional fetal RAS. Alternatively, rodent embryos may be able to utilise ANG II derived from the uterus, an organ shown to produce immunoreactive ANG II (De Silva et al. 1988). ANG II may be present in the exudate surrounding the early postimplantation conceptus which is derived from glandular secretions, the breakdown of decidual cells and the transudate of maternal serum. ANG II may therefore be transported intact across the rat yolk sac, a structure which is highly pinocytotic, to interact with embryonic ANG II receptors.

The ANG II evoked effects on a majority of the primordia studied, with evidence for a cephalocaudal gradient in response. Among these effects were those on the yolk sac and allantois. Compared with culture in retenate alone, ANG II stimulated development of the vitelline circulation and promoted the development of the 2 allantoic vessels which will form the umbilical vessels. The establishment of a fetoplacental circulation, and synthesis of the embryonic and fetal haemoglobins (initiated in the yolk sac) are vital to development. Angiotensinogen mRNA is detectable in rat yolk sac placenta from about 11 d gestation (Lee et al. 1987) and in very high concentrations in human yolk sac from 4–5 wk gestation (Schutz et al. 1996) while ACE is also present in rat trophoblast (Shi et al. 1997). Prorenin mRNA appears not to have been studied in the rat in early gestation, but prorenin concentrations in first trimester human villous placental tissue are very high (Downing et al. 1995). Thus the effects of exogenous ANG II in vitro reported in this study may be mimicking those occuring in vivo. Focal adhesion kinase (FAK) expression is especially abundant in the developing vasculature in the mouse embryo. Enhanced tyrosine phosphorylation of FAK has been described in response to ANG II (Polte et al. 1994). Thus ANG II may well be a factor implicated in placental and systemic vascular development from the very earliest stages.

ANG II also caused highly significantly improved development of the eye, forelimb bud and mandibular process, areas shown to express ANG II binding sites during late gestation (Millan et al. 1989). An intraocular RAS has now been detected in the rat (Berka et al. 1995) and human eye, particularly in the areas of greatest vascularity (Wagner et al. 1996) suggesting that the RAS may be an important regulator of eye function and it is possible that this may develop during gestation.

In order to mediate any growth promoting effects during organogenesis, ANG II must interact with its specific receptors; ANG II binding sites are detectable in the embryo from at least d 10 of gestation (Jones et al. 1989). The majority of ANG II receptors are of the AT2 subtype during gestation (Grady et al. 1991; Tsutsumi et al. 1991; Feuillan et al. 1993) and are expressed in many tissues such as undifferentiated mesenchyme which are not ANG II target tissues in the adult (Grady et al. 1991). In order to determine the role of the AT1 and AT2 receptor subtypes during organogenesis, embryos were cultured in the presence of GR117289 (AT1 blocker) and PD123319 (AT2

blocker). Culture in the presence of these antagonists in whole serum did not result in detrimental embryonic development, suggesting that the interaction of locally generated ANG II with these receptors is not essential for normal development. Similar findings have also been demonstrated in mice carrying disruptions of AT1 and AT2 receptor genes. Mice with a targeted disruption of the AT2 receptor gene develop normally and are healthy and fertile; however in postnatal life they demonstrate several behavioural abnormalities such as attenuated exploratory behaviour (Ichiki et al. 1995) and an impaired drinking response to water deprivation (Hein et al. 1995). Mice with a targeted disruption of the AT1a receptor also develop normally but exhibit decreased blood pressure and hyperreninaemia (Sugaya et al. 1995) and disruption of the genes encoding other components of the RAS, ACE and angiotensinogen, result in hypotension and renal abnormalities but normal embryonic development (Krege et al. 1995; Esther et al. 1996).

A significant increase in growth was however observed during retenate culture in the presence of physiological concentrations of ANG II. It may well be that the RAS is one of the hormone systems for which a 'fail-safe' system exists if the system is disrupted. To determine the receptor subtype mediating this response, embryos were cultured in the presence of antagonists in retenate culture supplemented with ANG II. Addition of the AT2 receptor antagonist PD123319 completely abolished the ANG II-induced increase in development observed in ANG II-supplemented retenate culture.This is in keeping with a variety of studies (see above) indicating that the predominant ANG II receptor in the early fetus is of the AT2 type. Shanmugam et al. (1995) identified AT2 mRNA expression from at least d 13, evolving and extending from a series of paired spots extending para-axially. The ANG II-induced increase in somite number was completely blocked by coculture with PD123319, which may reflect this same distribution. Again, the development of the forelimb involves apoptosis as well as growth and differentiation. The stimulatory effect of ANG II was blocked by PD123319. It is the AT2 receptor which appears to mediate the apoptotic effects of ANG II (Yamada et al. 1996). It is interesting that in this in vitro system culturing whole embryos, AT2 receptors were evidently maintained. The predominant ANG II receptor in freshly isolated rat fetal fibroblasts is the AT2 form, as it is in freshly prepared human fetal mesangial cells, but in culture AT1 receptors dominate (Johnson & Aguilera, 1991; Ray et al. 1994).

In the presence of GR117289 the growth of embryos cultured in retenate was significantly improved. This may be due to blockade of AT1 receptors by GR117289 resulting in the unmasking of other types of angiotensin receptor; endogenous ANG II present in the retenate (Table), unable to bind to AT1 receptors due to competitive inhibition by GR117289 could then bind to the unmasked AT2 or other receptors. Exogenous ANG II added to retenate culture under these circumstances would also activate unmasked receptors. Unmasking of the effects of ANG II at low concentrations by specific antagonists has been previously observed. In rabbit abdominal aorta the inhibition of AT2 receptors unmasked a low concentration effect of ANG II later discovered to be due to the action of AT1 receptors (Hong et al. 1994). An alternative explanation to the increase in embryonic development observed in the presence of GR117289 may be that embryonic AT1 receptors mediate growth inhibition and so blockade of AT1 receptors results in blockade of this function and causes increased growth. Some classical growth factors have also been shown both to stimulate and inhibit cell growth, for example transforming growth factor beta (Lee & Han, 1990). To attempt to determine which of these 2 theories was relevant, embryos were exposed to PD123319 and GR117289 simultaneously. This demonstrated that although the ANG II-induced increase in somite number and morphological score was not significantly reduced by the combined antagonists a significant increase in protein content was observed. This may suggest that the AT1 receptor has a growth inhibitory function during embryonic development, such that blockade of embryonic AT1 receptors results in increased growth. It is also possible that another type of ANG II receptor may be unmasked by this blockade of the AT1 and AT2 receptors, for example the AT3 receptor. This receptor appears not to have been studied in the embryo. Thus during embryonic development in retenate there may be a balance of growth promotion and inhibition caused by ANG II depending on receptor subtype activated.

This study has, for the first time, demonstrated a direct growth promoting effect of ANG II during organogenesis in the whole rat embryo. A role for ANG II as a growth factor during gestation may not be vital as demonstrated in the present study and in many gene disruption experiments. It is however possible that ANG II may play a more modest role during development, one that is only observed when embryonic development is impaired such as during retenate culture.

ACKNOWLEDGEMENTS

C.T. was supported by a grant from the Anatomical Society, to whom we express our thanks. We are also most grateful to Dr P. J. J. Admiraal, Rotterdam, for measurement of angiotensin II in whole rat serum and retenate, and to Glaxo Wellcome Research and Development, Stevenage, UK for the gift of GR117289.

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