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# Expression of the IGF System in Human Adrenal Tissues from Early Infancy to Late Puberty: Implications for the Development of Adrenarche

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## ABSTRACT

IGF-1, IGF-2, and type 1 IGF receptor (IGF-R1) mRNA expression and immunolocalization and cell proliferation index were studied in human adrenals from early infancy to late puberty. Adrenals were obtained from transplantation donors or from necropsies of endocrinologically normal subjects. Subjects were divided into three age groups: group 1, <3 mo of age, involution of fetal adrenals; group 2, 3 mo to 6 y of age, preadrenarche; and group 3, older than 6 y up to 20 y of age, postadrenarche. Cell proliferation index (Ki-67) in the outer, subcapsular, zona glomerulosa was significantly higher than in zona fasciculata of all groups and in zona reticularis or fetal zone. IGF-1 mRNA (semiquantitative reverse transcriptase–PCR and Northern blot) in group 2 was significantly higher than in group 1 and group 3 ($p < 0.05$). IGF2 mRNA in group 1 was significantly higher than in the other groups. IGF-R1 mRNA in group 3 was significantly higher than in group 2 but not different from group 1. Strong IGF-1, IGF-2, and IGF-R1 immunostaining signal was observed in the outer, subcapsular, zona glomerulosa and in zona fasciculata in the three groups, whereas a very weak IGF-1 and IGF-R1 immunostaining signal was found in fetal zone cells of group 1 and in zona reticularis of group 3. We propose that IGF-1 could be a factor involved in the postnatal mechanism of progenitor adrenal cell proliferation and migration. Our data also suggest that IGF-1 is not a direct regulatory factor of adrenal androgen production by zona reticularis cells. **(*Pediatr Res* 58: 451–458, 2005)**

**Abbreviations**

**CPI,** cell proliferation index
**DHEA,** dehydroepiandrosterone
**DHEAS,** dehydroepiandrosterone sulfate
**FeZ,** fetal zone
**IGF-R1,** type 1 IGF receptor
**RT-PCR,** reverse transcriptase–PCR
**ZF,** zona fasciculata
**ZG,** zona glomerulosa
**ZR,** zona reticularis

Adrenarche occurs only in higher primates, typically at ~6–8 y of age in humans, when adrenal zona reticularis (ZR) development takes place. This is an event of postnatal sexual maturation in which there is an increase in the secretion of adrenal androgens, mainly dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), not accompanied by an increase in cortisol secretion (1). Although several hypotheses have been suggested, the mechanism of this phenomenon remains unknown, suggesting that adrenarche might be a multifactorial event (2–4).

The adrenal gland is a dynamic organ. The regulation and function of rat and primate fetal adrenal gland (5–7) have been subjects of considerable interest and have been very useful for the understanding of tissue remodeling of the adrenal cortex. For most of gestation, the human fetal gland is characterized by rapid growth, high steroidogenic activity, and a unique morphology composed primarily of two zones: the inner fetal zone (FeZ) and the outer definitive zone. The large FeZ is the site of synthesis of DHEA and DHEAS, secondary to lack of 3$\beta$-HSD type 2 gene expression (8). The definitive zone, which is a thin cell layer, contains tightly packed cells with a proliferation phenotype that persists throughout gestation, and it does not acquire mineralocorticoid synthesis capacity until late in gestation. A third zone, the transitional zone, develops between

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the definitive zone and FeZ around midgestation, and it expresses enzymes that are required for cortisol synthesis (8). Soon after birth, the FeZ undergoes remodeling and involution. It has been proposed (5) that definitive zone cells may contain a progenitor cell population, some of which might migrate centripetally to populate transitional zones and FeZs. Moreover, it has been proposed (5) that some definitive zone cells persist into neonatal life, providing precursor cells for adult zones. However, it is less clear whether a centripetal migration model is entirely consistent with the growth and maintenance of adult adrenal cortex. Studies performed in 21-OH-ase/$\beta$-gal transgenic mice (9) have suggested that cells that originate from a peripheral multipotent stem cell population are displaced centripetally through the zones, suggesting, in turn, that zone-specific function results from phenotypic modulation of these cells in response to their position within the cortex (9). However, no information about a mechanism of remodeling of human adrenal gland is available when the development of the ZR takes place, at adrenarche. The molecular mechanism that is involved in the centripetal migration of definitive zone adrenal cells in the fetus to populate the remainder adrenal cortex and to initiate differentiation remains unknown. It was proposed recently that the recognition of matrix components by the integrin receptor, present at the plasma membrane, leads to the recruitment of multiple cytoskeleton-associated proteins and the subsequent activation of intracellular pathways leading to cell growth, survival, differentiation, and motility (10). On this line, it has been described that the abundance of IGF-2 mRNA is higher than that of IGF-1 mRNA, in the human fetal adrenal. In addition, IGF-2 is expressed in all corticotropin (ACTH)-responsive cortical cells, suggesting that IGF-2 is involved in the regulation of adrenal cortex steroidogenesis. However, at midgestation, when the zonation process becomes evident, IGF-1 is expressed only under the capsule (11,12). In contrast, in human adult adrenal cortex, IGF-2 mRNA expression is very low but IGF-1 mRNA is readily detected (12). Type 1 IGF receptor (IGF-R1) mRNA is also expressed abundantly in the definitive zone, transitional zone, and FeZ of the rhesus monkey fetus, but its expression decreases to undetectable levels at term (13). No information is available on the ontogenesis of IGFs and of IGF-R1 in human adrenal cortex from the neonatal period throughout adulthood.

We proposed recently that the GH/IGF axis might be an important metabolic signal involved in the maturational changes of human adrenal at the time of adrenarche, mainly in normal girls, and we have speculated that serum IGF-1 is necessary for the development of the ZR of the adrenal. Our data suggested that peripheral metabolic signals might be different in the two sexes (14,15). In the present work, we have studied the ontogenesis of IGF-1, IGF-2, and IGF-R1 mRNA expression and immunolocalization, as well as a cell proliferation index (CPI), in human adrenal tissues from early infancy to late puberty.

## METHODS

***Tissue samples.*** Forty-one nonpathologic human adrenal glands were obtained from multiorgan transplantation donors with <24 h after the diagnosis of brain death or patients who underwent resection of the kidney plus adrenal because of renal neoplasm ($n = 18$) or from necropsies with <6 h of postmortem time ($n = 23$). For analysis of the results, subjects were divided into three age groups: group 1, <3 mo of age ($n = 12$), group 2, between 3 mo and 6 y of age ($n = 17$); and group 3, older than 6 y up to 20 y of age ($n = 12$). This division was based on the mean age of postnatal FeZ involution (16) for the first cutoff and on the mean age of adrenarche (1) for the second. In group 3, only samples in which a continuous ZR was present were included. Table 1 shows data from all patients.

Segments of adrenals were immediately either fixed in 4% paraformaldehyde in PBS for 12–24 h and embedded in paraffin or promptly frozen and stored in liquid nitrogen for subsequent RNA analysis. The adrenal glands used in this study were histologically normal under the light microscope. The relevant clinical information and the pathologic studies were analyzed in every case, and samples from patients with endocrinologic disorders or congenital malformations of the midline of the brain were excluded. Data about the hormonal milieu of the patients were not available. Moreover, for addressing the functional capacity of the adrenal samples, four primary cultures of human adrenal cortical cells that were isolated from patients of different ages and from different sources (10 d old, necropsy; 2 mo old, necropsy; 5 mo old, necropsy; and 15 y old, organ donor) were carried out. As expected, mean basal cortisol secretion was increased 8.75 ± 3.98-fold by the addition of ACTH (10 nM) for 24 h ($p < 0.05$). The study was approved by the Ethical Committee of the Garrahan Pediatric Hospital.

***RNA isolation.*** Total RNA was extracted from each adrenal tissue sample by homogenization in TRIzol reagent (Invitrogen, Buenos Aires, Argentina) following the manufacturer's instructions. The integrity of each sample was checked by the presence of intact ethidium bromide–stained 28s and 18s ribosomal RNA bands. The purity of RNA samples was assessed by the 260/280 ratio (between 1.6 and 1.9) and by the absence of bands corresponding to contaminating DNA in agarose electrophoresis. RNA concentration was assessed by spectrophotometric absorbance at 260 nm.

*Semiquantitative reverse transcriptase–PCR of IGF-1, IGF-2, and IGF-R1.* Total RNA was reversely transcribed using Moloney murine leukemia virus reverse transcriptase (Amersham Biosciences, Buenos Aires, Argentina) following the manufacturer's instructions. The reverse transcriptase (RT) products then were amplified by PCR. The sequences of oligonucleotide primers were published previously (17–20). Each primer pair was localized on different exons to discriminate the products from genomic DNA and cDNA. Human $\beta$-actin gene, which is expressed at stable levels regardless at age (4), was used as a housekeeping gene to control PCR reactions and to normalize mRNA levels of IGF-1, IGF-2, and IGF-R1. Optimal PCR cycles that were required for linear amplification for each primer set were determined. Total amplification in each reaction ($\beta$-actin plus IGF-1, $\beta$-actin plus IGF-2, or $\beta$-actin plus IGF-R1) was kept below saturation levels. In the case of IGF-1 and IGF-R1, 0.12 $\mu$M of sense primer, 0.08 $\mu$M of 5'-end $^{32}$P-labelled sense primer, and 0.2 $\mu$M of antisense primer was added at the start of PCR. In the case of IGF-2, 0.2 $\mu$M each of the forward and reverse IGF-2 primers was added. The thermocycler was programmed for five cycles for IGF-1, eight cycles for IGF-2, and 12 cycles for IGF-R1; then $\beta$-actin primers were added and cycling was continued for an additional 20, 25, and 18 cycles, respectively.

The amplified products were separated by electrophoresis on 5% acrylamide 7 M urea gels for $^{32}$P-labeled PCR or on 2% gels stained with ethidium bromide for unlabeled PCR and visualized by autoradiography or under UV light. The intensities of the bands were quantified by densitometric analysis and normalized to that of $\beta$-actin (Molecular Image System, Bio-Rad GS-505; Bio Rad Laboratories, Hercules, CA). Each sample was analyzed at least twice, in duplicate, at two different dilutions, each time.

As negative control, the cDNA templates were substituted by an equal volume of distilled water and amplified in parallel. To evaluate the presence of the contaminating genomic DNA in the RNA-extracted preparation, a PCR reaction, in which the cDNA was replaced with the corresponding quantity of extracted total RNA, was performed without obtaining any amplification. The identity of RT-PCR products was verified by sequence analysis. Within- and between-assay coefficients of variation were 11.4 and 13.6, 14.3 and 15.5, and 10.9 and 12.8% for mRNA abundance of IGF-1, IGF-2, and IGF-R1, respectively.

Because approximately half of the patients died after severe systemic disease, the endocrine environment before death might have influenced adrenal function. For this reason, we compared mRNA abundance in samples of group 2 collected from necropsies ($n = 9$) or from organ donors ($n = 8$). No significant difference in IGF-1, IGF-2, or IGF-R1 mRNA abundance was found between the two different sources of sample collection.

*Northern blot.* The method of Sambrook *et al.* (21) was followed. Thirty micrograms of total RNA was electrophoresed, transferred, and cross-linked to the membrane as described. After prehybridization, hybridization was carried out at 42°C for 12–16 h using labeled PCR fragments of human adrenal IGF-1 cDNA (399 bp), IGF-2 cDNA (334 bp), IGF-R1 cDNA (600 bp), and $\beta$-actin

**Table 1.** *Data of patients*

| Gr | Age (years) | Sex | Source of adrenal | Diagnosis |
|---|---|---|---|---|
| Gr 1 | 0,006 | F | necropsy | congenital heart disease |
| Gr 1 | 0,008 | M | necropsy | congenital heart disease |
| Gr 1 | 0,011 | F | necropsy | congenital heart disease |
| Gr 1 | 0,016 | F | necropsy | congenital heart disease |
| Gr 1 | 0,019 | M | necropsy | congenital heart disease |
| Gr 1 | 0,041 | F | necropsy | congenital heart disease |
| Gr 1 | 0,047 | M | necropsy | congenital heart disease |
| Gr 1 | 0,072 | F | necropsy | congenital heart disease |
| Gr 1 | 0,1 | F | necropsy | pulmonary failure |
| Gr 1 | 0,125 | M | necropsy | hepatic failure |
| Gr 1 | 0,166 | M | necropsy | Sepsis |
| Gr 1 | 0,166 | F | necropsy | pulmonary failure |
| Gr 2 | 0,25 | F | necropsy | Sepsis |
| Gr 2 | 0,375 | M | necropsy | Sepsis |
| Gr 2 | 0,417 | F | necropsy | Sepsis |
| Gr 2 | 0,417 | F | necropsy | fibrocystic disease |
| Gr 2 | 0,583 | M | necropsy | biliary duct malformation |
| Gr 2 | 1,83 | M | organ donor | |
| Gr 2 | 2 | F | necropsy | cardiomyopathy |
| Gr 2 | 2 | F | organ donor | |
| Gr 2 | 2 | M | organ donor | |
| Gr 2 | 2 | F | necropsy | cardiomyopathy |
| Gr 2 | 2,5 | F | resection of kidney | Willms Tumor |
| Gr 2 | 3 | M | organ donor | |
| Gr 2 | 4,7 | F | necropsy | biliary duct malformation |
| Gr 2 | 6 | M | necropsy | pulmonary failure |
| Gr 2 | 6 | F | organ donor | |
| Gr 2 | 6 | M | organ donor | |
| Gr 2 | 6 | F | organ donor | |
| Gr 3 | 7 | M | organ donor | |
| Gr 3 | 8 | M | organ donor | |
| Gr 3 | 8 | F | organ donor | |
| Gr 3 | 9 | M | necropsy | encefalopathy |
| Gr 3 | 10 | F | organ donor | |
| Gr 3 | 11 | F | necropsy | pancreatitis |
| Gr 3 | 12 | M | organ donor | |
| Gr 3 | 14 | M | organ donor | |
| Gr 3 | 15 | F | organ donor | |
| Gr 3 | 18 | M | organ donor | |
| Gr 3 | 20 | F | organ donor | |
| Gr 3 | 20 | M | organ donor | |

cDNA (524 bp) as probes. These probes were labeled by primer extension with the corresponding 3' oligonucleotide in the presence of [$\alpha^{32}$P]dCTP (3000 Ci/mmol; Amersham Bioscience). To remove nonspecifically bound probe, membranes were washed and subjected to autoradiography using Kodak XAR-5 x-ray film at −70°C. Complete removal of probe was confirmed by autoradiography before reprobing between consecutive hybridizations. Equal loading of RNA samples was confirmed by using a human $\beta$-actin $^{32}$P-labeled probe and by comparing with 28S rRNA.

***Immunohistochemistry of IGF-1, IGF-2, and IGF-R1.*** Immunohistochemistry staining was performed using the streptavidin-biotin and peroxidase method according to the manufacturer's protocol (DAKO Catalyzed Signal Amplification System, HRP; DAKO Cytomation, Carpinteria, CA). Briefly, after deparaffinization, sections (5 $\mu$m) were subjected to antigen retrieval and proteinase K treatment. Endogenous biotin activity was blocked and endogenous peroxidase activity was quenched. The sections were blocked further with a protein block for 15 min to decrease nonspecific staining. Sections then were incubated with the primary antibody for IGF-1 (5 $\mu$g/mL, KJH-I01 Gro Pep), IGF-2 (1 $\mu$g/mL, sc-5622; Santa Cruz Biotechnology, Santa Cruz, CA), or IGF-R1 (2 $\mu$g/mL, MAB1120; Chemicon International, Temecula, CA) for 18 h at 4°C. After washing, tissues were incubated for 15 min with biotinylated goat anti-rabbit (IGF-1 and IGF-2) or biotinylated rabbit anti-mouse (IGF-R1) immunoglobulins. Bound antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride, which results in a brown-colored precipitate at the antigen site. Counterstaining was performed using hematoxylin, which stains cell nuclei blue. As negative controls, normal rabbit serum (for IGF-1 and IGF-2) or normal mouse serum (for IGF-R1) was used instead of primary antibodies. No specific immunoreactivity was detected in these sections. Experiments were repeated twice, and there were no differences of the patterns of immunolocalization between the two experiments.

**In situ *detection of cell proliferation.*** For localizing proliferating cells, a monoclonal mouse anti–Ki-67 (Clone Ki-S5, Code M7187; DAKO Cytomation) was used. Slices were pretreated as described above. The sections were incubated with mouse anti–Ki-67 (1:25 dilution) for 1 h at room temperature. As a negative control, the specific antiserum was replaced by normal mouse serum. Immunostaining was performed with the streptavidin-biotin and peroxidase method (DAKO Catalyzed Signal Amplification System) as described above. Positive nuclei from cycling cells were stained brown, whereas nuclei from noncycling cells were staining blue (hematoxylin counterstaining). Proliferation index in different adrenal cortical zones was assessed by counting at least 1000 nuclei and determining the proportion (expressed as a percentage) of Ki-67–positive cells. Experiments were repeated at least twice.

***Statistical analysis.*** For statistical analysis, mRNA data, expressed in arbitrary units (AU), were square root–transformed so that values approximated a normal distribution. Differences between means of mRNA levels among age groups were evaluated by Bonferroni test after one-way ANOVA. Proliferation index difference between cortical zones was compared using paired $t$ test. Differences in mean proliferation index between groups was examined by Kruskal Walls test. A $p < 0.05$ was consider statistically significant.

## RESULTS

***CPI in human adrenal cortex zones in the three age groups.*** For each specimen, an hematoxylin-eosin staining was done to delineate histologically distinct glomerulosa, fasciculata, reticularis, and FeZ zones. Histologic identification of the zones of the adrenal cortex was based on previously published descriptions (22). Specifically, the glomerulosa was identified as discontinuous subcapsular aggregates of small cells with small nuclei and few lipid droplets. The fasciculata was identified as the large, lipid-laden cells arranged in a cord-like pattern. The reticularis was identified by the lack of cord-like organization and the compact and lipid-poor nature of the cells. The FeZ was identified as eosinophilic, lipid-rich cells located at the inner cortex, in glands collected from postnatal subjects (group 1) (23).

CPI (%) in group 1, group 2, and group 3 is shown in Table 2. Within groups, in the three age groups, CPI was significantly higher ($p < 0.05$) in the outer, subcapsular, zona glomerulosa (ZG) than in the zona fasciculata (ZF) and ZR, as well as in group 1 FeZ. It is remarkable that in the inner adrenal zones, such as in the FeZ and in ZR, the lowest CPI was found. However, among groups, mean CPI in similar or equivalent zones was not different.

Representative results of Ki-67 immunostaining signal in group 1, group 2, and group 3 adrenal tissues are shown in Fig. 1*A*, *B*, and *C*, respectively. The majority of Ki-67–positive cells are located in the outer, subcapsular, ZG cells in the three age group. In all age groups, Ki-67–positive cells decreased from the outer to the inner layers of the adrenal cortex. These results suggest that the proliferation cell layer is located in the outer subcapsular ZG.

***IGF-1, IGF-2, and IGF-R1 mRNA abundance and cell immunolocalization in human adrenal cortex in the three age groups.*** To evaluate whether the IGF system plays a role in adult adrenal remodeling and/or in the differentiation process of adrenal ZR cells, we assessed first the levels of IGF-1, IGF-2, and IGF-R1 mRNA and second the zonal distribution of IGF-1 and IGF-R1 in human adrenal tissues as a function of age. Measurement of IGF-1, IGF-2, and IGF-R1 mRNA abundance by semiquantitative RT-PCR (AU) in group 1, group 2, and group 3 is shown in Fig. 2. Mean (± SD) IGF-1 mRNA value in group 2 (1.64 ± 0.88) was significantly higher than in group 1 and group 3 ($p < 0.05$; Fig. 2*A*). However, in group 1 infant, in whom some FeZ cells persist in most tissues, and in group 3 children and adolescents, in whom ZR was present, similar mean values were found (0.88 ± 0.27 and 0.88 ± 0.30, respectively). Mean ± SD IGF-2 mRNA abundance is shown in Fig. 2*B*. In group 1, mean IGF-2 mRNA was significantly higher (1.99 ± 0.38) than in the other two groups ($p < 0.05$), but no significant difference was found between group 2 (1.43 ± 0.38) and group 3 (1.30 ± 0.16). Mean ± SD IGF-R1 mRNA abundance is shown in Fig. 2*C*. In contrast to IGF-1 mRNA expression, IGF-R1 mean mRNA increased significantly at the age when adrenarche takes place (group 3). However, means in group 1 and group 3 were similar (group 1, 3.93 ± 2.0; group 2, 1.98 ± 0.9; and group 3, 3.7 ± 1.44; $p < 0.05$).

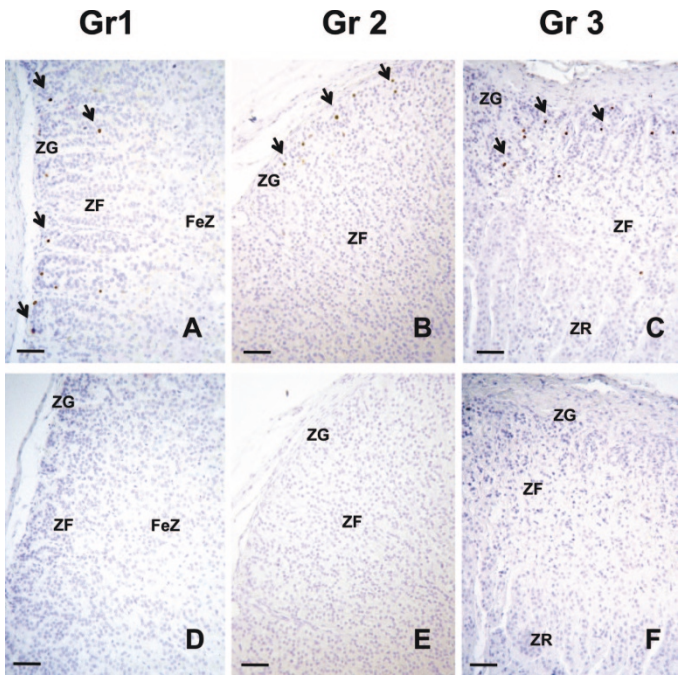


**Figure 1.** Immunohistochemical detection of Ki-67 antigen (brown staining in top panels) in sections of normal human adrenal gland of group 1 (*A*), group 2 (*B*), and group 3 (*C*). Arrows indicate examples of positive staining for Ki-67. The highest labeling was found in the ZG. The control of each group exhibited no signal (*D–F*). FeZ, fetal zone. Bars = 250 μm.

In addition, Northern blot analysis was performed to confirm the presence and the size of IGF-1, IGF-2, and IGF-R1 mRNA in adrenal tissues. Arbitrarily, two samples per age group were selected (Fig. 3). Three major species of IGF-1 mRNAs (7.7, 5.3, and 0.9 Kb) and two IGF-R1 mRNA transcripts (11 and 7 kb) were expressed in human adrenal tissues. There was a marked increase in the expression of IGF-1 mRNA in group 2. In contrast, adrenal glands from group 2 tended to have less IGF-R1 mRNA than those from group 1 and group 3. mRNA encoding IGF-2 was readily detected in total RNA from human

**Table 2.** *Cell proliferation index (number of Ki-67-positive cells-% of total cells-) in each zone of human adrenal cortex*

| Age group | Ages (yr) | Glomerulosa | Fasciculata | Reticularis | Fetal |
|---|---|---|---|---|---|
| Gr1 | < 3 month (n = 12) | 10,47 ± 6,43* | 3,78 ± 3,09 | | 0,53 ± 0,40† |
| Gr2 | 3 month–6 (n = 17) | 15,41 ± 8,22* | 5,32 ± 2,79 | | |
| Gr3 | > 6–20 (n = 12) | 13,51 ± 6,38* | 4,67 ± 3,00 | 2,01 ± 1,86** | |

Data expressed as mean ± SD.
* glomerulosa compared with fasciculata within each age group, p < 0,05.
** reticularis compared with fasciculata in Gr3, p < 0,05.
† fetal zone compared with fasciculata in Gr1, p < 0,05.

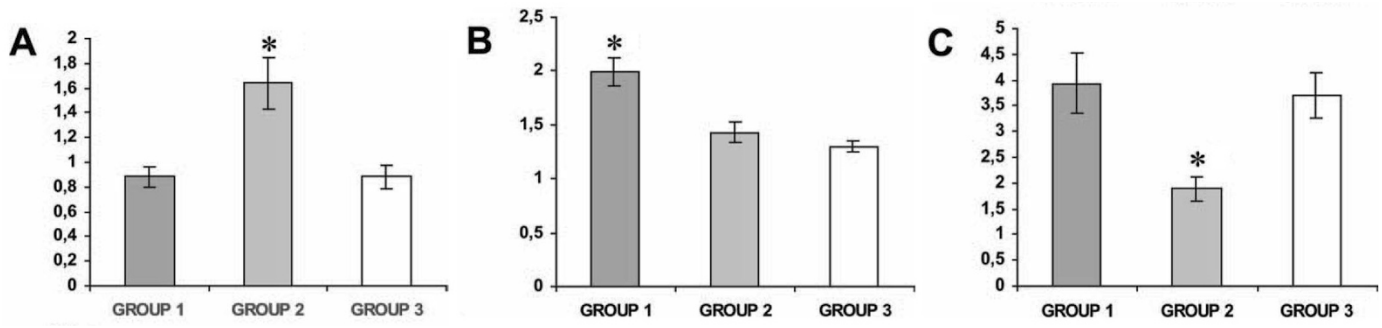


**Figure 2.** Semiquantitative RT-PCR mRNA expression of IGF-1 (*A*), IGF-2 (*B*), and IGF-R1 (*C*) in normal human adrenal gland. Group 1, $n = 12$; group 2, $n = 17$; and group 3, $n = 12$. The bars show mean mRNA levels, quantified densitometrically in relation to β-actin mRNA (AU); the error bars represent SEM in each group. Results are expressed as mean ± SEM; $*p < 0.05$.

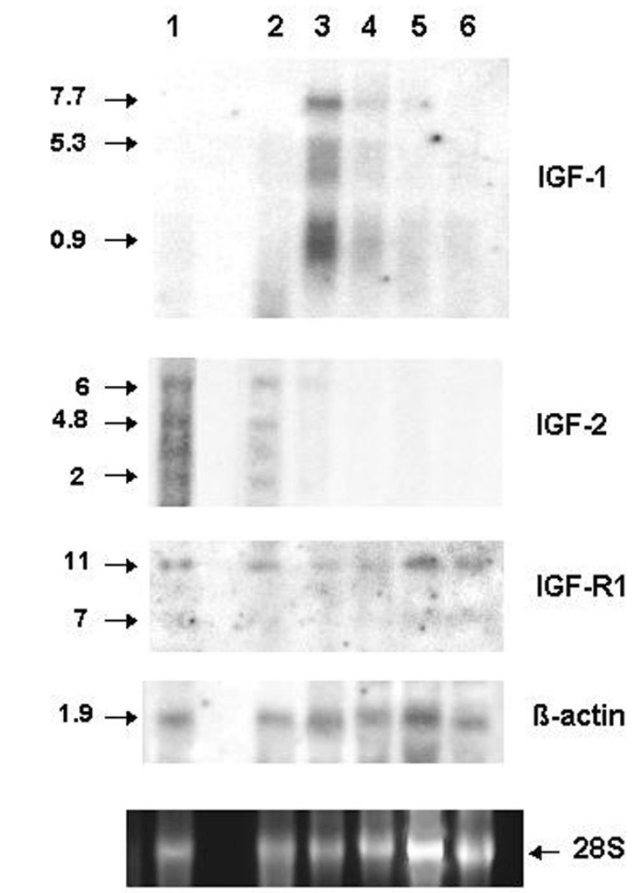


**Figure 3.** Northern blot analysis. Thirty micrograms of total RNA was sequentially hybridized with IGF-1, IGF-2, and IGF-R1 probes, with a β-actin probe as a loading control. Lanes 1 and 2, human adrenal RNA of group 1 (15- and 26-d-old subjects, respectively); lanes 3 and 4, human adrenal RNA from group 2 (3-mo- and 3-y-old subjects, respectively); lanes 5 and 6, human adrenal RNA from group 3 (11 and 15 y of age, respectively). The lower panel is a picture of the ethidium bromide–stained gel showing rRNA abundance. Sizes are in kb.

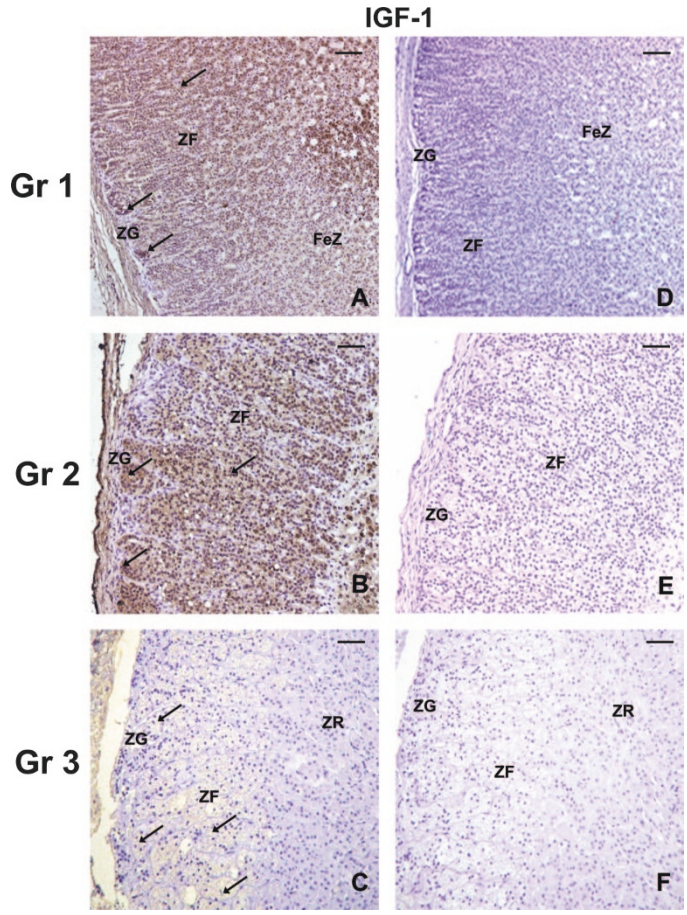


**Figure 4.** Immunohistochemical localization of IGF-1 in sections of normal human adrenal gland of group 1 (*A*), group 2 (*B*), and group 3 (*C*). Immunopositive cells appear brown as a result of 3,3'-diaminobenzidine tetrahydrochloride colorimetric reaction. Arrows indicate heavily stained regions. Hematoxylin is used as the nuclear stain. The control of each group (*D–F*) exhibited no signal. Bars = 500 μm in *A* and *D*; 250 μm in *B*, *C*, *E*, and *F*.

adrenal glands of group 1 but was barely detectable in tissues from group 2 and group 3. Several IGF-2 mRNA transcripts were found. The major bands were 6, 4.8, and 2 kb, although some additional bands were also seen.

Representative results of IGF-1 cell immunolocalization in group 1, group 2, and group 3 are shown in Fig. 4*A*, *B*, and *C*, respectively. In group 1, group 2, and group 3, a strong IGF-1 immunostaining signal in the outer, subcapsular ZG and in ZF cells was observed. However, similar to the FeZ of group 1, in ZR cells of group 3, a very weak IGF-1 immunostaining signal was observed, suggesting that in cells with androgen synthesis capacity, the expression of IGF-1 is very low. IGF-2 immunostaining (data not shown) was observed in the three age groups. In all of them, maximal intracellular staining was evident in ZG and decreased toward FeZ. In group 1, important IGF-2

expression was found in FeZ and in the FeZ. In group 3, diffuse weak and mild staining in ZF and in ZR, respectively, was present.

Representative results of IGF-R1 cell immunolocalization in group 1, group 2, and in group 3 are shown in Fig. 5*A*, *B*, and *C*, respectively. In the three age groups, immunostaining signal cell distribution was similar to that of IGF-1. Even though immunohistochemistry is not a reliable quantitative method, in group 3, a relative increment of IGF-R1 immunostaining signal was observed in the outer, subcapsular ZG cells when compared with group 2.

## DISCUSSION

For appropriate validation of the study, 41 tissue samples were selected carefully from >120 adrenals. Even though using data from necropsy tissues is a major concern in this study, the relevant clinical information and the pathologic studies were available and checked in every case. Specimens from patients with endocrinologic disorders or congenital malformations of the brain were excluded, as well as samples that revealed signs of mRNA degradation. In four primary cultures of the adrenal tissues, mean basal cortisol secretion was significantly increased by ACTH, indicating that they maintained glucocorticoid functional capacity. Patients' diagnoses were evenly distributed among groups. Even though it has been reported that in patients who had severe life-threatening illnesses at the time of death (24,25) or in brain-dead potential organ donors (26,27) serum ACTH and cortisol levels are controversial, ACTH overstimulation *in vivo* could not be discarded. In a previous study of 3$\beta$-HSD mRNA expression in human adrenal tissues that we have published (4), tissue samples were obtained from similar sources. Results observed in this study were similar to those published by Gell *et al.* (3) and Wan *et al.* (28). Furthermore, the importance of human adrenal samples is stressed by the fact that ZR cells from animals are not morphologically and functionally homologous to human ZR.

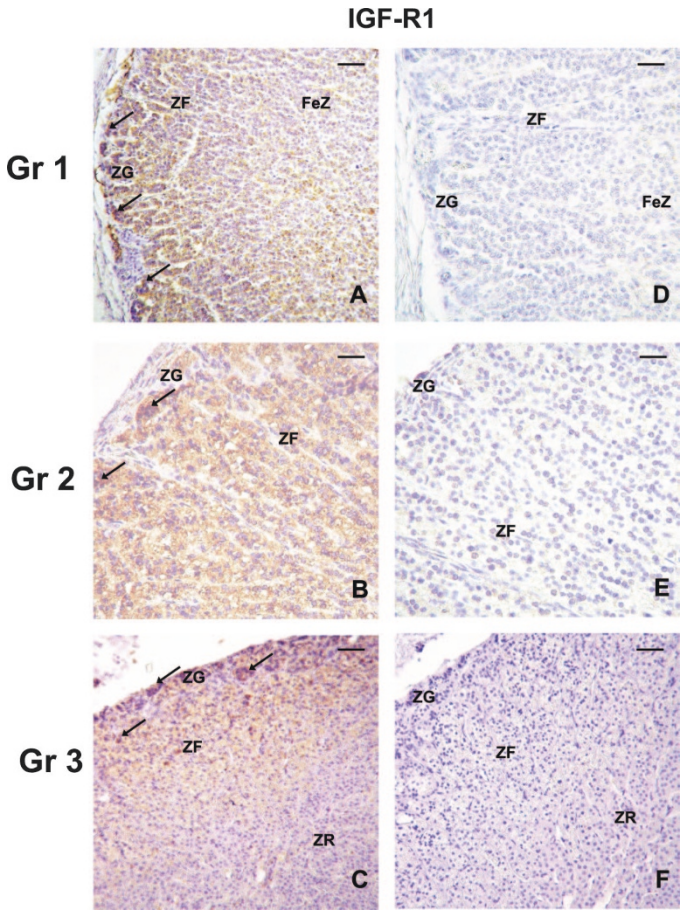


**Figure 5.** Immunohistochemical localization of IGF-R1 in sections of normal human adrenal gland of group 1 (*A*), group 2 (*B*), and group 3 (*C*). Immunopositive cells appear brown as a result of 3,3'-diaminobenzidine tetrahydrochloride colorimetric reaction. Arrows indicate heavily stained regions. Hematoxylin is used as the nuclear stain. The control of each group (*D–F*) exhibited no signal. Bars = 250 $\mu$m in *A*, *C*, and *F*; 125 $\mu$m in *B*, *D*, and *E*.

Because cell proliferation and programmed cell death play important roles in the maintenance of tissue dynamics, we became interested in the study of the CPI in our adrenal tissues. We found that this index was clearly higher in the outer zone of the human adrenal gland, mainly below the adrenal capsule, in all age groups studied. Within each age group, ZF CPI was almost 3-fold less than in the periphery of the gland. Moreover, in the FeZ of the youngest group, as well as in ZR of the oldest one, CPI was low. These results are in agreement with the findings described in the human fetal adrenal cortex, once the adrenal zonation process starts (29). Even though the role of the definitive zone in human fetal adrenal cortex is unknown, it has been proposed that its cells may comprise a progenitor population, some of which migrate centripetally to populate transitional zone and FeZ (5). Mitani *et al.* (30) described, in rat adult adrenal cortex, a new zone that is in between ZF and ZG with an undifferentiated cell phenotype and that did not respond by cell replication, to ACTH, suggesting that cytogenesis in this region was regulated by a mechanism other than ACTH. As far as we know, no study of cell proliferation of the human adrenal cortex during childhood and puberty has been published. In contrast to the adult adrenal cortex rat model described by Mitani *et al.* (30) but similar to definitive zone cells in the human fetal adrenal cortex (5), our CPI study showed that during childhood and adolescence, adrenal progenitor cells are probably located in the outer zone of the adrenal cortex. Therefore, we suggest that this peripheral cell layer, below the capsule, contains the undifferentiated progenitor cells.

The molecular mechanisms involved in adrenal progenitor cell proliferation, migration, and functional differentiation remain poorly understood, and it seems logical to propose that multiple factors are playing a role. Laser capture microdissection has been used to identify putative developmental genes as markers to identify progenitor, intermediate, and mature cell types (31). It has been proposed that IGF-1 is a key growth factor in fetal development. It is a potent mitogen and a promoter of cell migration of normal and tumor cells. These effects of IGF-1 are mediated by the IGF-R1, a member of the receptor tyrosine kinase family. It has been also proposed that the key aspect of cell growth and motility is the capacity of IGF-1 to promote tyrosine phosphorylation of focal adhesion

proteins (32,33). Hence, in this study, we have hypothesized that peripheral or local IGF-1 could be involved in the mechanism of progenitor cell proliferation and migration. Therefore, we studied the ontogenesis of IGF-1, IGF-2, and IGF-R1 expression in human adrenal tissues in the three age groups. To analyze this particular issue, we developed two different approaches. First, we analyzed the abundance of IGF-1, IGF-2, and IGF-R1 mRNAs by semiquantitative RT-PCR and confirmed these findings by Northern blot analysis. Second, we analyzed cell localization of the corresponding proteins by immunohistochemistry. IGF-1 mRNA abundance was similar in the youngest group, in which the FeZ is still present in most adrenals, and in the oldest group, in which ZR has been developed. However, in the intermediate age group, IGF-1 mRNA expression was higher than in the other two groups. The highest IGF-1 mRNA expression in the intermediate age group could be secondary to a dilution effect of IGF-1 mRNA, because the cell type component in the other two groups was different. Indeed, IGF-1 immunohistochemistry studies revealed a weak signal, not only in FeZ cells but also in ZR cells. Similar to definitive zone cells of human fetal adrenal cortex (13), a strong IGF-1 signal in the outer, subcapsular, ZG cells of the adrenal cortex was found in the three age groups. However, in contrast to human fetal adrenal cortex, a strong IGF-1 signal in ZF was found in the three age groups. Therefore, we can speculate that in the human adrenal cortex, from early infancy to late puberty, IGF-1, expressed in the outer, subcapsular ZG cells, might stimulate adrenal progenitor cell proliferation and migration. However, in ACTH-responsive cells, such as ZF cells, IGF-1 might be involved in the regulation of adrenal steroidogenesis. In this regard, it has been described that IGFs increase basal and ACTH-induced steroidogenesis, in human adult adrenal cells in culture. In addition, IGF-1 induces specific steroidogenic enzyme genes, such as 3$\beta$-HSD and 17$\alpha$-hydroxylase (34–36).

IGF-2 has been implicated as an important regulator of fetal growth, but the role of IGF-2 during postnatal life is less clear. We have found that IGF-2 mRNA expression was higher in the youngest group and that it decreased later on. Immunohistochemical analysis revealed high expression in the three zones of group 1 but particularly under the capsule. In the periphery of the gland, the expression persisted in the other two groups, as well as in ZR of group 3. For these reasons, we cannot discard a role of IGF-2 in postnatal adrenals. A postnatal effect of IGF-2 has been suggested by Wolf *et al.* (35) in transgenic mice.

Even though it has been reported that IGF-2 had a stronger effect than IGF-1 on cortisol and DHEAS stimulation in human adrenal cells in culture, the effect of IGFs was confirmed to be mediated by IGF-R1 (34). Moreover, it has been postulated that the activation of IGF-R1 by IGFs or by high levels of insulin is implicated in the developmental regulation of adrenal androgen secretion, as well as in the pathogenesis of various states of hyperandrogenemia (36,37). In relation to the role of the IGF system as a major regulator of adrenal androgens, we found that IGF-R1 mRNA expression increased from the intermediate to the oldest study group. However, immunohistochemistry revealed a very weak signal in ZR cells, suggesting that the IGF system is not involved directly in the regulation of adrenal androgens by ZR cells. Moreover, in the residual FeZ cells of the youngest group, a pattern similar to that of ZR cells was found. Even though IGF-R1 immunostaining in ZF cells was detected in the three age groups, in every case the strongest signal was found in the outer, subcapsular, ZG cells. As IGF-R1 mRNA increased from group 2 to group 3, we propose that, for the development of ZR during childhood, this receptor positively modulates the proliferation and migration to ZR of adrenal progenitor cell located under the capsule. However, the factors that are involved in these phenomena must be elucidated.

## CONCLUSION

In summary, we propose that IGF-1 and perhaps IGF-2, by autocrine, paracrine, or endocrine stimulation, could be a factor involved in the postnatal mechanism of progenitor adrenal cell proliferation and migration. Our data also suggest that IGF-1 is not a direct regulatory factor of adrenal androgen production by ZR cells, although further studies are necessary to clarify the relationship between the IGF system and adrenal function.

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