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Human Adrenal Cells Express Tumor Necrosis Factor-α Messenger Ribonucleic Acid: Evidence for Paracrine Control of Adrenal Function*

JOSÉ A. GONZÁLEZ-HERNÁNDEZ†, MONIKA EHRHART-BORNSTEIN, ERNST SPÄTH-SCHWALBE, WERNER A. SCHERBAUM, AND STEFAN R. BORNSTEIN

Department of Internal Medicine III, University of Leipzig (J.A.G.-H., M.E.-B., W.A.S., S.R.B.), Leipzig; and the Department of Internal Medicine III, University of Ulm (E.S.-S.), Ulm, Germany

ABSTRACT

Tumor necrosis factor (TNF) is gaining increasing importance in clinical medicine. It plays a role in the interaction of the immune system with the hypothalamic-pituitary-adrenal axis. In the present study various morphological methods, including immunohistochemistry, electron microscopy, and *in situ* hybridization were applied to characterize the localization and distribution of TNF in the human adrenal gland.

Double immunostaining revealed an astonishing degree of intermingling of steroid-producing cells and chromaffin cells. Macrophages could be found in all regions of the adrenal gland, but particularly in the transition zone of cortex and medulla. The steroid-producing cells of the inner zone of the cortex express major histocompatibility complex class II molecules. On the ultrastructural level, immune cells, steroid cells, and catecholamine-producing cells were found in direct contact. The combination of immunohistochemistry and *in situ* hybridization was optimally suited to define the exact cellular source of TNF in the human adrenal. TNF is produced in macrophages, but above all in 17α-hydroxylase-positive cells (steroid-producing cells) in the zona reticularis and medulla. No signal was found in chromaffin cells. TNF may induce major histocompatibility complex class II in human adrenal gland in a paracrine or autocrine manner. It is concluded that TNF may have an important role in normal human adrenal physiology. (*J Clin Endocrinol Metab* **81:** 807–813, 1996)

TUMOR NECROSIS factor (TNF) is a cytokine produced primarily by macrophages, monocytes, natural killer cells, and astrocytes in response to infectious and inflammatory stimuli. TNF has numerous activities, including mediation of cachexia, cytotoxic effects against tumor cells, induction of antiviral activity, and activation of macrophages (1). The data concerning the beneficial therapeutic effect of TNF and TNF antibodies in different pathological conditions remain controversial (2–4). However, recent data suggest that TNF-related therapies may play a major role in the near future in clinical medicine (5–7). Particularly in light of this development, it appears important to define the normal physiology of this cytokine in humans. Recent reports by several groups suggest that TNF may serve as an important link between the neuroendocrine and immune systems (8, 9). Activation of the immune system by exposure to antigens leads to increased secretory activity of the hypothalamic-pituitary-adrenal axis (10). This response is mediated by the release by monocytes and mononuclear cells of cytokines, particularly interleukin-1 (IL-1), IL-6, and TNFα, which act directly and indirectly on the hypothalamus to stimulate the synthesis and secretion of CRH. This hormone, in turn, stimulates the release of corticotropin and, thereby, of cortisol (11, 12). According to recent data, TNF may also stimulate steroidogenesis by inducing the release of ACTH from pituitary cells (13, 14). On the other hand, direct effects, both stimulatory and inhibitory, of TNF on adrenal cells have been reported (15–18).

The presence of major histocompatibility complex (MHC) class II antigenic determinants within the normal adrenal gland has been observed (19). In many tissues, the expression of MHC class II on cells is controlled by cytokines. TNF is a powerful inducer of MHC antigen expression on many cell types that would otherwise express MHC molecules only weakly (20). If TNF occurs in the adrenal, it may induce the expression of MHC class II in adrenal cells.

Cytokines synthesized within endocrine organs have an important role as local tissue regulators of hormone production (21). These cytokines are secreted locally in most tissues, where they exert paracrine regulatory effects in response to circulating toxins and cytokines. The results of recent studies demonstrated that adrenal cells may produce several cytokines, including IL-1 and IL-6, both of which are able to influence steroidogenesis (22–24). In addition, measurable levels of TNF were found in fetal human adrenals (17), but to date it is not known whether TNF is expressed in the adult human adrenal gland.

In various mammalian, particularly human, adrenals, steroid-producing cells and chromaffin cells are intermingled to an astonishing degree (25–27). In addition, macrophages oc-

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Address all correspondence and requests for reprints to: Dr. S. R. Bornstein, Department of Internal Medicine III, University of Leipzig, Philip Rosenthal Straße 27, 04103 Leipzig, Germany.

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† Present address: Department of Applied Clinical Physiology, Hermanos Ameijeiras Hospital, University of Havana, Havana, Cuba.

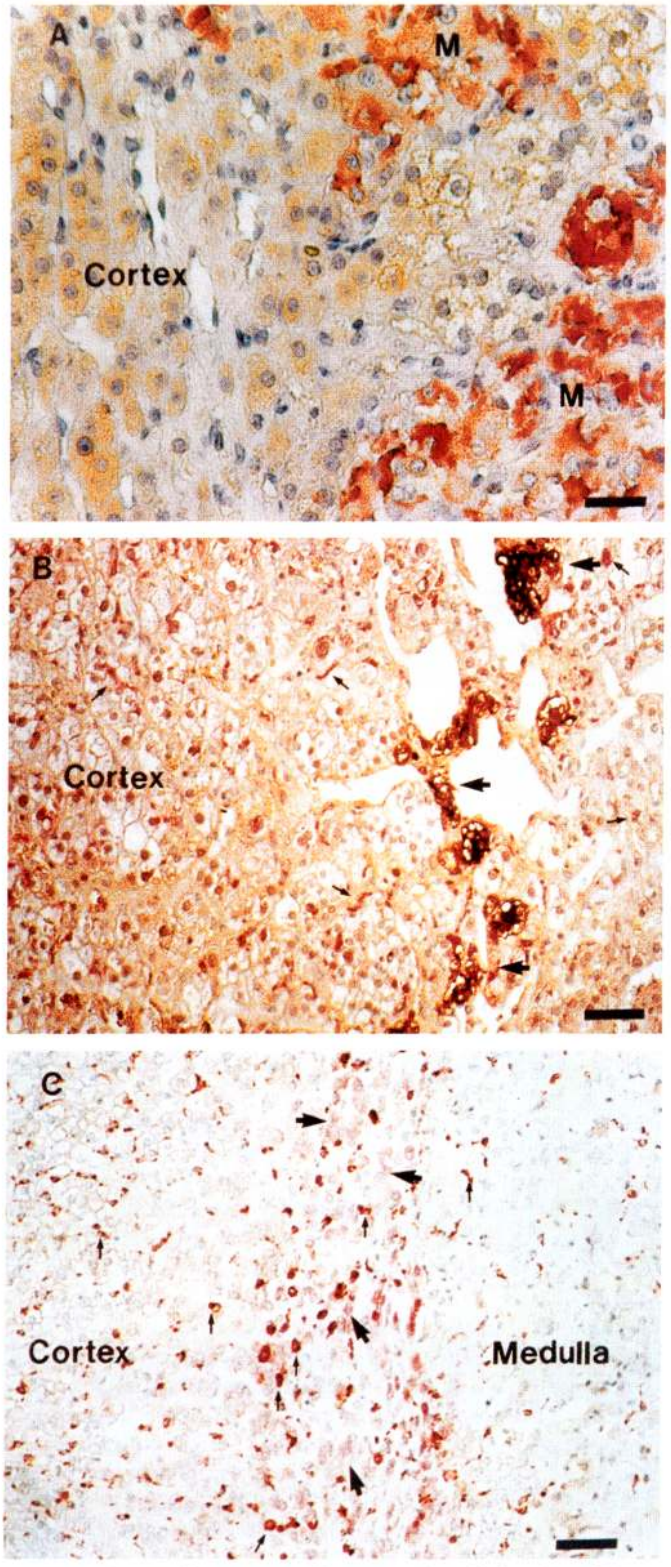

FIG. 1. A–C, Paraffin sections of human adrenal gland. Double immunostaining was employed to study the different cell types within the adrenal. A, A great degree of intermingling of chromaffin cells (labeled red with antihuman chromogranin A and the APAAP method) and cortical cells (labeled brown with anti-17α-hydroxylase cytochrome P450 enzyme and the PAP method) is shown (magnification, ×66; *bar* = 150 μm). M, Medulla. B, The macrophages (labeled red with anti-CD68 antigens and the APAAP method) occurred throughout the gland, principally in fasciculata and reticularis zones (*small arrows*); the chromaffin cells were also identified by brown labeling with antichromogranin A and the PAP method (*large arrows*; magnification, ×45; *bar* = 204 μm). C, The MHC-class II molecule (labeled red with anti-MHC class II molecule and the APAAP method) is expressed by cells in the transitional zone of cortex and medulla (*large arrows*) and by macrophages in all regions (labeled with anti-CD68 antigens combined with anti-MHC class II antigens; *small arrows*; magnification, ×45; *bar* = 204 μm).

cur in all zones of the gland (28). Therefore, each cell type was characterized by specific immunostaining. With the help of double immunohistochemistry, the distribution of the various cell types could be defined, and the MHC class II-positive cells could be identified. To determine whether the different cell types are in direct contact, the cells were analyzed by electron microscopy. Ultimately, the combination of *in situ* hybridization and immunohistochemistry was optimally suited to define the cell types that express TNF in the normal human adrenal gland.

Material and Methods

Normal adrenal glands were obtained from eight human subjects who underwent nephrectomy due to renal carcinomas. The age of the donors varied between 40–56 yr. This investigation was approved by the ethical committee of the University of Leipzig. The donors had no signs or symptoms of adrenal disorder and had taken no medication before surgery. Adrenal tissues were fixed in Bouin's solution for 24 h and embedded in paraffin. Some of the paraffin sections were stained with hematoxylin and eosin.

Immunohistochemistry

Deparaffinized sections (4 μm) of human adrenals were separately immunostained using the unlabeled peroxidase-antiperoxidase (PAP) method (29). Adrenocortical cells were immunostained for 17α-hydroxylase cytochrome P450. The mouse monoclonal antibody was kindly provided by Prof. Waterman (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN). To identify MHC class II molecule, we used an antibody (mouse monoclonal, Dakopatts, Hamburg, Germany) that reacts with the β-chain of all products of the gene subregions DP, DQ, and DR.

Slides were preincubated with 5% rabbit serum in Tris-buffered saline (TBS; 0.1 mol/L; pH 7.6) and exposed to the specific antiserum at 4 C overnight. The antisera were used at dilutions of 1:100 in 5% normal rabbit serum in TBS, washed in TBS (three times, 10 min each time), and incubated with the second antiserum (rabbit antimouse IgG; Dakopatts) at a dilution of 1:50 for 60 min in TBS. After being washed in TBS, the sections were incubated with the mouse PAP complex (Dakopatts) at the same dilutions as the primary antibody for 30 min in 5% normal rabbit serum in TBS. Visualization was achieved by immersing the sections in 0.05% (wt/vol) 3′,3-diaminobenzidine (Sigma Chemical Co., Munich, Germany) and 0.01% H_2O_2 in Tris-HCl, pH 7.6, for 10 min. Slides were stained with hematoxylin, rinsed in water, dehydrated, and mounted. As controls, the specific antisera were replaced by nonimmune rabbit serum. These control sections showed no unspecific staining.

Double immunohistochemistry

The sections were first stained by a three-step peroxidase technique, as described above, followed by detection of the second antigen using the alkaline phosphatase-antialkaline phosphatase (APAAP) method, as previously described (23). Briefly, peroxidase-stained sections for cortical cells, adrenomedullary cells (antihuman chromogranin A, mouse monoclonal antibody, Dakopatts), and macrophages (anti-CD68, mouse monoclonal antibody, Dakopatts) were sequentially incubated with antichromogranin A, anti-CD68, and anti-MHC class II antibodies, respectively, and with APAAP complex (Dianova, Hamburg, Germany). Alkaline phosphatase activity was visualized using naphthol-AS phosphate and Fast red after blocking of endogenous alkaline phos-

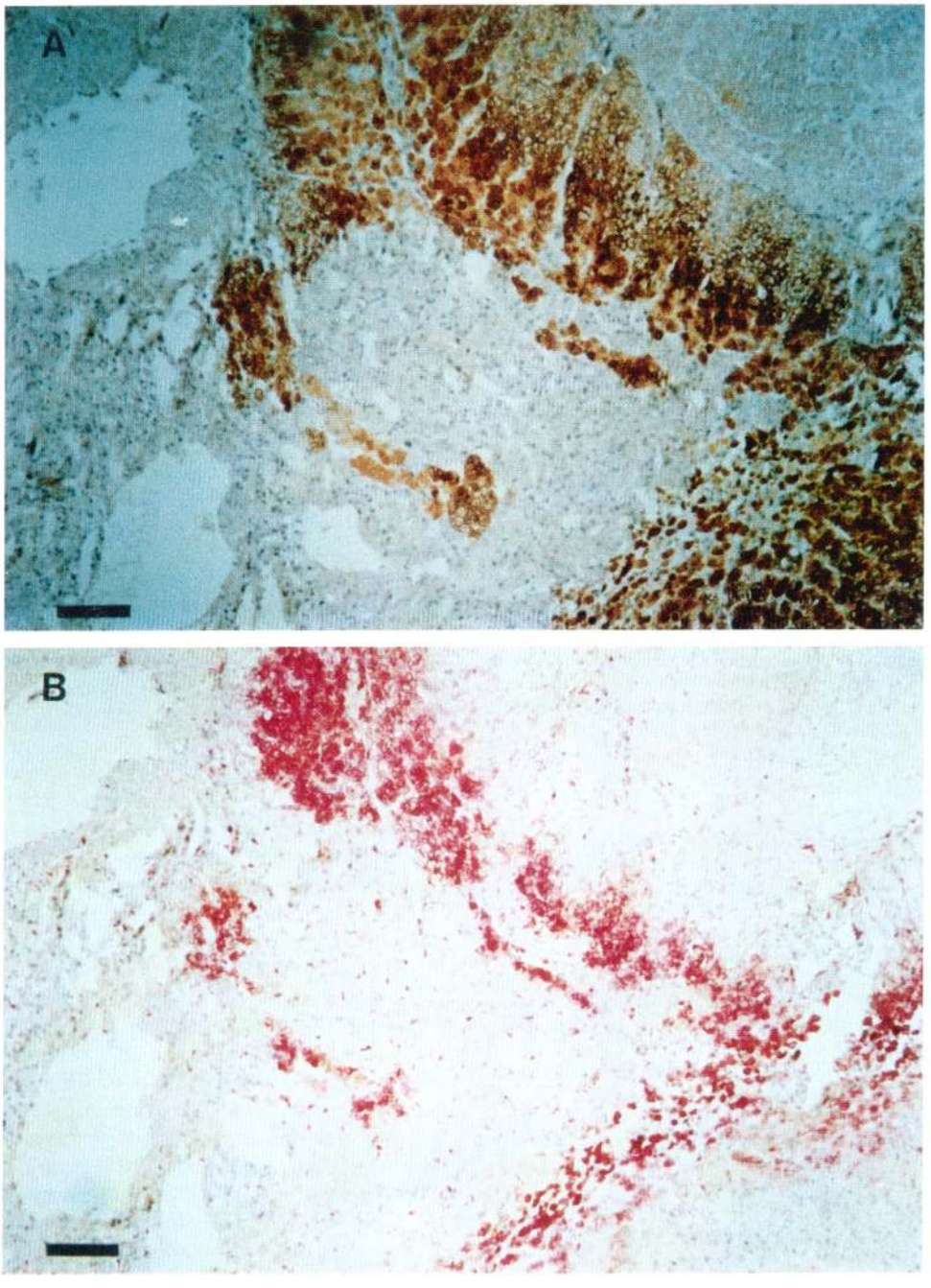

FIG. 2. A and B, Sequential paraffin sections of adrenal gland. A, The 17α-hydroxylas7e-positive cells (steroid-producing cells, labeled brown) that coexpress MHC class II antigen (B; labeled red) are located principally in the zona reticularis, with increasing density towards the medulla (magnification, ×33; *bar* = 300 μm).

phatase with Levamisol (Sigma). Appropriate controls were performed to exclude cross-reactivities.

In situ hybridization

In situ hybridization was performed as described previously (23). The sections were deparaffinized through xylene and graded ethanol (100%, 95%, 90%, 80%, and 70%) and immersed for 5 min in distilled water supplemented with 0.1% diethylpyrocarbonate (Sigma). The sections then were rinsed in 0.1 mol/L triethanolamine, pH 8.0 (Sigma), containing 0.25% acetic anhydride (Sigma) for 15 min at room temperature, washed in 2 × standard saline-citrate buffer (SSC) twice for 2 min each time, dehydrated through graded ethanol, and dried at room temperature in a dust-free atmosphere. The synthetic oligonucleotide used in this study is specific for human TNFα and corresponds to complementary sequences (antisense) of the messenger ribonucleic acid (mRNA). The corresponding sense probe was used as a control. The specificity of this oligonucleotide has been described previously (30). The probe has been radiolabeled with [^{35}S]deoxy-ATP (DuPont, Boston, MA) by using terminal deoxynucleotidyl transferase (Promega Corp., Heidelberg, Germany) according to the manufacturer's protocol. The labeled probe was purified by Sephadex G-25 gel filtration (Pharmacia, Freiburg, Germany), and radioactivity was measured in a scintillation counter. The

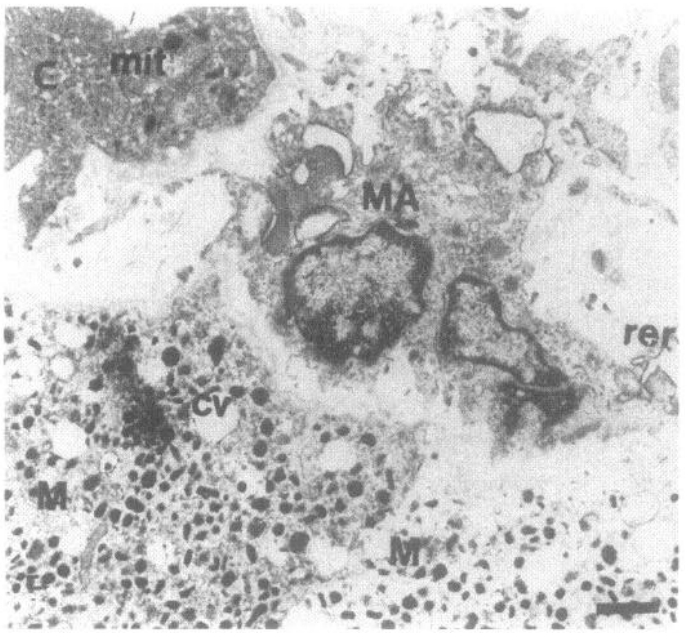

FIG. 3. Electron micrograph of human adrenal gland. Macrophages (MA) are frequently located in the transition zone of medulla (M) and cortex (C). A macrophage is in direct contact with a chromaffin cell (M), recognized by the chromaffin vesicles (CV). An adrenocortical cell (C) is also in direct apposition with the macrophage (MA). Adrenocortical cells are characterized by their typical tubulo-vesicular mitochondria (mit; magnification, ×32,400; *bar* = 0.3 μm). rer, Rough endoplasmic reticulum.

specific activity of the probe ranged from 75,000–100,000 cpm/μL. The concentration of the probe was adjusted to approximately 10,000 cpm/μL in a hybridization mix consisting of 50% formamide, 5 × Denhardt's solution, 4 × SSC, 10% dextran sulfate, 20 mmol/L sodium phosphate (pH 7.0), and 10 mg/mL denatured salmon sperm DNA. Finally, dithiothreitol was added to the mixture at a final concentration of 5 mol/L. Sections were incubated with the probes overnight at 40 C in a humid chamber. Slides were washed sequentially in 2 × SSC at room temperature three times for 5 min each time, 1 × SSC at room temperature twice for 15 min each time, 0.1 × SSC at 50 C for 15 min, and 0.1 × SSC at room temperature shortly to cool the slides down. Thereafter, the slides were dehydrated through graded ethanol (50%, 70%, and 100%), dried, and coated with autoradiography emulsion (Ilford K.5, Dreieich, Germany), air-dried, and exposed for 4 weeks in the dark at 4 C. Finally, the slides were developed in D-19 solution (Eastman Kodak, Rochester, NY) for 4 min, fixed in express fixing salt (Tetenal Photowerk, Neuheim, Germany) for 4 min, and counterstained with hematoxylin for 2 min. Control experiments included the hybridization with labeled TNFα sense probe.

Combined in situ hybridization and immunohistochemistry

In the first step, *in situ* hybridization was performed. After hybridization, slides were washed, and tissue sections were processed for immunostaining. Finally, the slides were processed and developed as described above.

Electron microscopy

For ultrastructural investigations, small tissue slices of four glands were fixed in a fixative containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.3, for 3 h. The tissue slices were postfixed for 90 min (2% OsO_4 in 0.1 mol/L cacodylate, pH 7.3), dehydrated in ethanol, and embedded in epoxy resins. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined at 80 kV in a Phillips electron microscope 301 (Phillips Electronics, Rahway, NJ).

Results

Double immunostaining was used to study the presence and distribution of different cell types within the adrenal gland. Sections in which chromaffin and cortical cells were specifically immunostained revealed a great degree of intermingling of the two endocrine tissues (Fig. 1A). The CD68-positive cells (macrophages) occurred throughout the gland, predominantly in the zona fasciculata and zona reticularis. In these zones, chromogranin A-positive cells (chromaffin cells) were also found (Fig. 1B). The expression of MHC class II antigens was widely distributed within the adrenal gland. Staining was prominent in cells of the zona reticularis, with increasing density toward the medulla. In the zona fasciculata and zona glomerulosa, stained cells were seen scattered throughout. Double immunohistochemistry identified many of the MHC class II-positive cells as tissue macrophages that were distributed throughout the entire gland (Fig. 1C). When sequential immunostaining for 17α-hydroxylase cytochrome P450 enzyme and MHC class II antigens was performed, it became clear that steroid-producing cells were also able to express MHC class II molecules, particularly in the zona reticularis (Fig. 2, A and B). At the ultrastructural level, macrophages were found in direct apposition with cortical and chromaffin cells. Macrophages were characterized by their typical lysosomes at different developmental stages and their pseudopods (Fig. 3).

Cells expressing TNFα mRNA were detected by *in situ* hybridization using a ^{35}S-labeled antisense oligonucleotide probe. Individual cells showing strong signals after hybridization were localized within the zona fasciculata (Fig. 4A). Cells containing TNF were also scattered in the zona reticularis, with increasing density toward the zona medullaris.

Simultaneous detection of TNFα mRNA and immunohistochemistry for either CD68 or 17α-hydroxylase were performed to further characterize the cytokine-producing cells. Most of the TNF-expressing cells were immunoreactive for 17α-hydroxylase cytochrome P450 enzyme (Fig. 4B). Single cells that hybridized in the adrenal medulla also stained for 17α-hydroxylase (Fig. 4C). In contrast, chromaffin cells were in all cases devoid of TNF. A fraction of the radiolabeled cells in zona reticularis were CD68 positive (Fig. 4, D and E). This suggests that in the adrenal gland, adrenocortical cells and/or macrophages may be responsible for TNF production *in vivo*. Serial sections showed that 17α-hydroxylase-positive cells in the zona reticularis coexpressed TNFα mRNA and MHC class II antigens. The hybridization with control probes (sense) resulted only in nonspecific binding of radioactive material.

Discussion

TNF is a pleiotropic cell regulatory protein produced mostly by monocytes and macrophages; among its several functions, it is involved in the cytokine-mediated communication between the immune system and the hypothalamic-pituitary-adrenal axis. This is the first study to demonstrate the precise cellular sources of TNF in the human adrenal gland. As the various cell systems in this tissue, including tissue macrophages, cortical cells, and chromaffin cells, were found to be intermingled to an astonishing degree, the com-

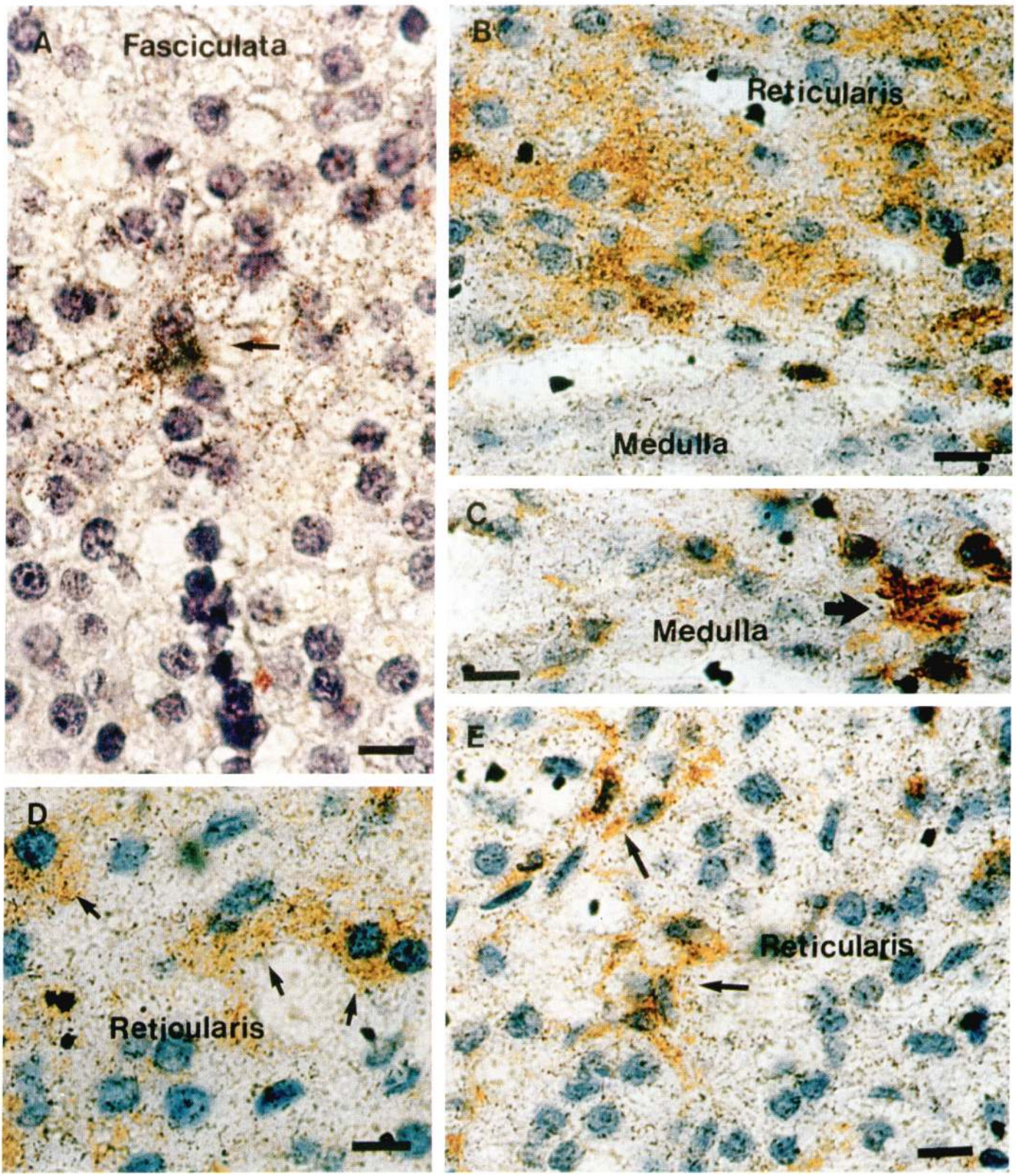

FIG. 4. A–E, Expression of the TNFα mRNA in paraffin sections of the human adrenal gland. A, A strong signal is apparent after hybridization with the antisense probe in single cells of the zona fasciculata (magnification, ×400; *bar* = 25 μm). The combination of *in situ* hybridization and immunohistochemistry permits the visualization of the TNFα mRNA and the exact identification of its cellular source. B, The majority of cells expressing TNFα (silver grain) in zona reticularis are steroid-producing cells (labeled brown with anti-17α-hydroxylase cytochrome P450 and the PAP method; magnification, ×330; *bar* = 30 μm). C, TNFα mRNA-positive signal also occurs in cells within the zona medullaris (*arrow*), but they were clearly identified as steroid-producing cells by labeling brown with anti-17α-hydroxylase and the PAP method (magnification, ×330; *bar* = 30 μm). D and E, Single macrophages immunostained brown with anti-CD68 antigen (PAP method) coexpress TNFα mRNA, in particular within the zona reticularis (*arrows*; D: magnification, ×400; *bar* = 25 μm; E: magnificaiton, ×330; *bar* = 30 μm).

bination of immunohistochemical staining of each cell type with *in situ* hybridization was the only reliable method to define the exact cellular source of TNF production. The 17α-hydroxylase cytochrome P450-positive cells were found to be predominantly responsible for TNF synthesis in the human adrenal gland. A small number of cytokine-producing cells were identified as resident macrophages, preferentially located in the zona reticularis. Cells expressing the TNFα mRNA were especially frequent in the transitional areas of the adrenal that contain cortical cells, macrophages, and chromaffin cells. TNF expression also occurred within the adrenal medulla. Surprisingly, the cytokine was not located in chromaffin cells, but was detected in cortical cells that occur within the medulla. This again underlines the importance of double staining for a correct interpretation of the results. The ultrastructural studies showed that close physical contact between these different cells is a common feature. Such contact could provide the basis for paracrine interactions between these cells.

There is increasing evidence that local mechanisms are involved in the regulation of adrenocortical function. For example, TNF is released from the rat adrenal zona glomerulosa (31, 32), it can modify the release of steroids directly (32), and it may be an important negative modulator of aldosterone synthesis (18). The local release of TNF is stimulated by lipopolysaccharide and IL-1 (32). On the other hand, in addition to the direct effect that TNF may have on adrenal function, this cytokine may modify adrenal steroid secretion by stimulating the local release of IL-6 (31). As we have previously reported the expression of IL-1 and IL-6 in human adrenal gland *in vivo* (23, 24), we would tentatively propose/hypothesize that all of these adrenal cytokines may interact in a complex manner to regulate each other's secretion and may thereby modify adrenal function. Jaattela *et al.* (16) previously demonstrated that fetal human adrenals produce TNF and that this cytokine inhibits the ACTH-induced production of cortisol. Other studies from adult animals (15, 32) show that TNF increases the release of cortisol. As the expression of TNF and its consequent modulation of adrenal steroidogenesis appear to depend on the developmental state of the adrenals, we suggest that TNF may be an important factor in the local coordination of the development and function of this organ.

The possibility of a stress-mediated cytokine expression has to be considered due to the fact that adrenal glands in this study were taken from endocrinologically normal subjects who underwent nephrectomy. However, as all patients had normal levels of plasma cortisol and ACTH before surgery (data not shown), it is likely that TNF is a local regulator of adrenal function under normal conditions. Data from other steroid tissues, such as the ovary, show that local production of TNF is involved in physiological processes (21, 33).

Circulating and tissue levels of TNF are elevated in a variety of clinical situations and correlate with the severity of some diseases, suggesting that TNF participates in the host response to or development of illness (34). It is known that the endotoxin-induced glucocorticoid production is mediated by TNF (35). Such an effect of TNF on corticosteroid release seems to have a protective effect, as adrenalectomized animals are markedly sensitive to TNF-induced lethality (36, 37). Therefore, the local production of TNF in the adrenal gland may be important in this setting. On the other hand, if TNF occurs in excess amounts, it leads to severe toxicity and death. Soni *et al.* (38) reported clinical evidence of adrenal insufficiency in a subgroup of patients with septic shock and proposed that TNF may play a role in the development of this insufficiency, as serum TNF levels were inversely related to mean arterial pressure. However, if activation of the hypothalamic-pituitary-adrenal axis occurs either by endotoxin stimulation or during the stress response to different conditions, the decrease in adrenal TNF release after ACTH stimulation (32) may contrast with the peripheral overproduction of TNF by monocytes, in which TNF can be induced by ACTH (39). Another series of investigations has convincingly demonstrated that the lethality of TNF in rats is abrogated by TNF pretreatment with nonlethal doses (40). If the tolerance phenomenon also occurs in humans, in particular in the adrenal gland, a full understanding of the role of the locally produced TNF may be relevant for clinical applications and may help to improve survival and outcome in sepsis (*e.g.* meningococcemia).

It is known that chromaffin cells contain different neuropeptides, such as enkephalins, vasoactive intestinal polypeptide, neurotensin, and substance P among others (41, 42). The patterns of expression of these four neuropeptides are modified by exposure to TNF (41). However, the presence of TNF, either in the mature adrenal medulla or during adrenomedullary development, has not yet been reported. Therefore, the regulatory function of TNF on the production of neuropeptides by medullary chromaffin cells may be expected to be mediated by TNF released either from cortical cells in the cortex reaching the medulla through the blood flow or from cortical cells within the medulla.

An interesting finding in this study was that steroid-producing cells coexpress TNF and MHC class II antigens. TNF is one of the major regulator of the MHC class II expression *in vitro* and *in vivo* (20). Adrenocortical cells of the zona reticularis normally express MHC class II antigenic determinants (19). Therefore, a role of TNF in the expression of MHC class II molecules by adrenal cells could be expected. Moreover, the expression of MHC class II antigens on resident tissue cells may play a role in suppressing local immune responses (43). In this context, a protective effect of TNF in different autoimmune diseases has been postulated (44, 45). It could be that MHC class II and TNF have a protective role in the human adrenal. The expression of TNF in human adrenal cortical cells itself and the expression of MHC class II in the same cells underline the close link between the immune system and the adrenal gland.

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