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Immunohistochemical localization of adult T-cell leukaemia-derived factor, a human thioredoxin homologue, in human fetal tissues

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Summary. An immunohistochemical study of the expression of adult T-cell leukaemia-derived factor (ADF), a human thioredoxin homologue, was performed using a rabbit antibody against the C-terminal peptides of ADF. Tissues were obtained from human fetuses between 9 and 23 weeks of gestation. It was revealed that ADF was widely distributed in different organs and tissues during the fetal period. The ADF antibody reacted selectively with medullary cells of the thymus, lung epithelium, the epithelium of the digestive tract, hepatocytes, bladder epithelium, peripheral nerve cells, hair follicles, sebaceous glands, osteoblasts and the proximal tubules of the kidney. It also reacted with cells destined to differentiate into ciliated cells in the fallopian tube and efferent ductules of the testis, interstitial cells in the ovary, Leydig cells of the testis, and dendritic cells in the spleen and lymph nodes. This is the first report on the thioredoxin system in human cells during the early fetal period. The selectivity of ADF staining in fetal tissues suggests that, during early fetal life, ADF expression correlates well with the cellular function of certain tissues.

Key words: Adult T-cell leukaemia – Derived factor – Immunohistochemistry – Thioredoxin

Introduction

Human T-cell lymphotropic virus (HTLV)-1-transformed T-cells not only express high levels of interleukin-2 receptors [IL-2R/p55(Tac)], but also produce an IL-2R/Tac inducer designated the adult T-cell leukaemia (ATL)-derived factor (ADF) (Teshigawara et al. 1985; Tagaya et al. 1988). We have previously cloned the ADF cDNA and found that ADF production in human lymphocytes can be enhanced by cellular activators such as mitogens or phorbol esters (Tagaya et al. 1989, 1990). Recombinant ADF produced by *Escherichia coli* is a multi-functional protein with many biological activities such as the promotion of lymphocyte proliferation and synergism with IL-1 or IL-2, besides IL-2R/Tac inducing activity (Tagaya et al. 1989). A homology search revealed a close relationship between ADF and a dithiol-reducing enzyme, thioredoxin (Holmgren 1985; Tagaya et al. 1989, 1990). ADF is thus considered to be a human homologue of thioredoxin having a strong thiol-dependent reducing activity.

We recently synthesized a synthetic peptide incorporating the C-terminal 29 amino acids of ADF (ADF C-peptides) and an antibody against the peptide was raised in rabbits. Western blot analysis using this anti-ADF antibody revealed that ADF is expressed not only in HTLV-1-transformed cells (ATL-2), but also in Epstein-Barr virus-transformed cells (3B6) (Wakasugi et al. 1987) and human papillomavirus-transformed cells (HeLa) (unpublished observation). In addition, an immunohistochemical study of human tumours, such as cervical cancer (Fujii et al. 1991), hepatoma, and lung cancer has revealed that a large number of tumour cells are positive for ADF. Therefore, ADF is expressed not only by haematopoietic cells but also by certain neoplastic epithelial cells. This finding prompted us to study the localization of ADF in human fetal tissues to determine whether or not ADF is expressed during the fetal period.

Materials and methods

A synthetic peptide incorporating the C-terminal 29 amino acids of ADF (ADF C-peptide) was synthesized using an automatic peptide synthesizer (Applied Biosystems, Calif.) and an anti-ADF antibody was then raised against this synthetic peptide conjugated with bovine serum albumin (BSA). The conjugate was injected subcutaneously into rabbits together with Freund's complete adjuvant. After three immunizations, serum from the rabbits was purified by saturated ammonium sulphate precipitation, followed by application to a BSA-sepharose column to remove anti-BSA components. The serum was further purified using an immobilized ADF column. The specific antibody solution was dialysed against phosphate buffered saline and stored frozen at −20° C. Specificity of the antibody was tested by Western blotting using recombinant

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Table 1. Immunohistochemical reactivity of fetal and adult tissues for the anti-adult T-cell leukaemia-derived factor (ADF) antibody

	Gestational age (weeks) and sex					Adult
	Female			Male		
Number of fetuses	9 (2)	12–17 (6)	20–23 (4)	17 (1)	23 (2)	
Tissue examined						
1. Ectoderm-derived tissue						
Skin						
Squamous cell	−	−	−	−	−	−
Hair follicle			+		+	+
Sebaceous gland			+		+	+
Nervous tissue						
Central	−	−	−	−	−	NE
Peripheral	+	+	+	+	+	+
2. Endoderm-derived tissue						
Thyroid			+		+	+
Parathyroid			−		−	NE
Thymus						
Cortex	−	−	−	−	−	−
Medulla	+++	+++	+++	+++	+++	++
Respiratory system	+	+	+	+	+	+
Digestive tract	++	++	++	++	++	+
Liver	++	++	++	++	++	+
Gallbladder	+	+	+	+	+	NE
Pancreas	−	−	+	−	+	+
Urogenital sinus	−					
Urinary bladder		++	++	++	++	+
3. Mesoderm-derived tissue						
Bone and cartilage	−	−	−	−	−	−
(osteoblasts)	+	+	+	+	+	+
Muscle tissue	−	−	−	−	−	−
Spleen	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]
Lymph node	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]
Adrenal						
Cortex	+	+	+	+	+	+
Kidney						
Glomerulus	−	−	−	−	−	−
Proximal tubules	++	++	++	++	++	+
Female genital system						
Ovary (interstitial cells)		++	++			++[b]
Fallopian tube (ciliated cells)		++	++			+
Endometrium		+	+	−	−	+++[c]
Male genital system						
Leydig cells				++	++	++
Efferent ductules				+++	+++	NE

Reactivity for the anti-ADF antibody: strongly positive (+++), moderately positive (++), weakly positive (+), and negative (−)
[a] Dendritic cells were positive for ADF
[b] Granulosa, theca cells, and corpus luteum were positive for ADF
[c] Predecidual cells in the luteal phase and decidual cells during pregnancy were strongly positive for ADF
NE, Not examined

ADF, ADF C-peptide, and purified ADF obtained the conditioned medium of ATL-2 cells (Maeda et al. 1985).

Fresh specimens of various fetal tissues were obtained from human fetuses (12 female fetuses between 9 and 23 weeks of gestation, and 3 male fetuses at 17 and 23 weeks of gestation) following legal elective abortions. The tissues shown in Table 1 were used for the study and informed consent was obtained to study these fetuses.

Specimens of various adult normal tissues shown in Table 1 were obtained at surgery and were also used for the study.

For immunohistochemistry tissues were fixed in either formalin or Bouin's solution and embedded in paraffin. Thin sections were deparaffinized in toluene, and immunohistochemical staining was then performed as follows. Deparaffinized sections were treated with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, and incubated with normal goat serum to block non-specific antibody binding. Then sections were incubated with the primary antibody (rabbit anti-ADF IgG, 2.0 μg/ml) at 37° C for 60 min, or with normal rabbit serum for the negative controls. The sections were then treated with anti-rabbit IgG-biotin complex, followed by avidin-peroxidase complex, and stained with 3-amino-9-ethylcarbazole solution plus 0.15% hydrogen peroxide. Reagents except for the primary antibody were obtained from the Universal rabbit kit system (Biomeda, Foster City, Calif.). Counterstaining was performed with haematoxylin. HTLV-1-transformed cells (ATL-2) (Maeda et al. 1985) served as the positive controls. The intensity of staining for ADF was evaluated by staining the same specimens several times and the observation was performed by more than two observers. Intensity was graded as (−) for no staining, (+) for weak staining, (++) for moderate staining, and (+++) for strong staining. Histological observation of the specimens was performed using routinely processed sections stained with haematoxylin and eosin.

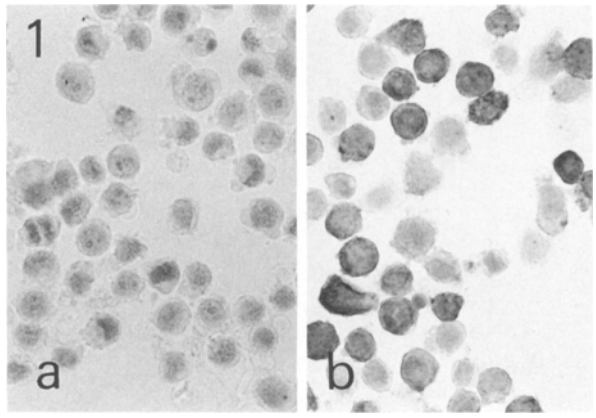


Fig. 1. Immunohistochemical staining of human T-cell lymphotropic virus (HTLV)-1-transformed cells (ATL-2) for adult T-cell leukaemia derived factor (ADF): **a** negative control; **b** positive staining of the cytoplasm. ×400

Results

A large number of ATL-2 cells (HTLV-1-transformed cells) had their cytoplasm stained red, indicating positivity for ADF (Fig. 1a, b).

In ectoderm-derived tissues (Table 1) in the fetus the squamous epithelium of the skin was negative for ADF, but hair follicles (Fig. 2a, b) and sebaceaus glands observed from 20 weeks of gestation onwards showed moderate reactivity for ADF. Nerve cells in the central nervous system were negative for ADF, but the cytoplasm of some ganglionic cells and peripheral nerve cells was weakly positive for ADF from 9 weeks of gestation.

In endoderm-derived tissues (Table 1) the follicular cells of the thyroid gland showed weak reactivity for ADF, while the parathyroid glands were negative. The cortex of the thymus was negative for ADF, but the medullary cells (particularly the epithelio-reticular cells which surround the thymic epithelioid cells) showed strong positivity for ADF from 9 weeks of gestation (Fig. 3). Hassal's corpuscles were negative for ADF (Fig. 3). In the respiratory system the bronchiolar epithelium showed weak reactivity for ADF. In addition, ciliated cells of the bronchus and trachea also showed weak positivity for ADF. In the digestive tract the endodermal epithelial lining including the oesophagus (Fig. 4a, b), stomach, intestines (Fig. 5a, b), and rectum showed moderate to strong reactivity for ADF from 9 weeks of gestation. In particular, the ciliated cells of the oesophagus (Fig. 4b) and the undifferentiated epithelial cells of the digestive tract showed moderate to strong positivity. However, mucus-secreting cells such as intestinal goblet cells were negative for ADF. Hepatocytes were positive for ADF (Fig. 6), but haematopoietic cells seemed to be negative. In the gallbladder the epithelium showed weak positivity for ADF, and both the acinar cells and the islet cells displayed weak reactivity for ADF in the pancreas. The epithelium of the urogenital sinus was negative for ADF at 9 weeks of gestation (Fig. 7a). However, the epithelium of the urinary bladder showed moderate reactivity for ADF by 20 weeks gestation (Fig. 7b).

Of the mesoderm-derived tissues (Table 1) both bone and cartilage were usually negative for ADF, although osteoblasts showed moderate to strong positivity (Fig. 8). Skeletal muscle, cardiac muscle, and smooth muscle were all negative for ADF. A few dendritic cells were positive for ADF, but other spleen cells were negative for ADF. Dendritic cells were positive for ADF in lymph nodes. The inner zone of the adrenal cortex was weakly positive for ADF (Fig. 9a, b), but the medulla was negative. The epithelial cells of the proximal tubules and collecting tubules of the kidney showed positivity for ADF (Fig. 10), with the proximal tubules reacting particularly strongly with anti-ADF. However, the glomeruli were negative.

In the female genital system both wolffian and müllerian ducts were negative for ADF at 9 weeks of gestation. Mesenchymal and epithelial cells of the uterus were also negative for ADF. However, in the fallopian tubes, ciliated cells observed from 20 weeks of gestation onwards showed strong positivity for ADF (Fig. 11). In the fetal ovary, we often observed interstitial cells which are believed to be identical to Leydig cells. These interstitial cells in the ovaries were selectively positive for at 16 and 23 weeks of gestation (Fig. 12), but the other constitutive cells of the ovary were negative for ADF. In the fetal testis, Leydig cells were selectively positive for ADF (Fig. 13a, b). In the epithelium of the efferent ductules, the cells which were destined to differentiate into ciliated cells showed strong reactivity for ADF (Fig. 14), but the other constitutive cells of the testis were negative.

Almost all adult tissues which reacted with the anti-ADF antibody during the fetal period also showed positivity for ADF (Table 1). However, the intensity of staining in these tissues was usually weaker than during the fetal period. The difference of ADF staining between the fetal and adult period was observed in the tissues of the female genital system, which show development

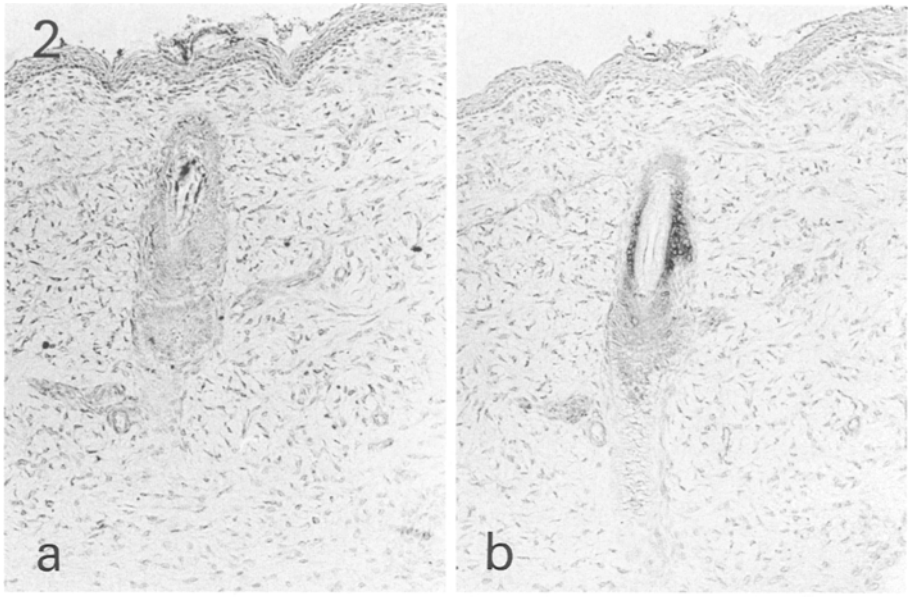


Fig. 2. Immunohistochemical staining of the fetal skin for ADF at 22 weeks of gestation: **a** negative control; **b** positive staining of a hair follicle. ×100

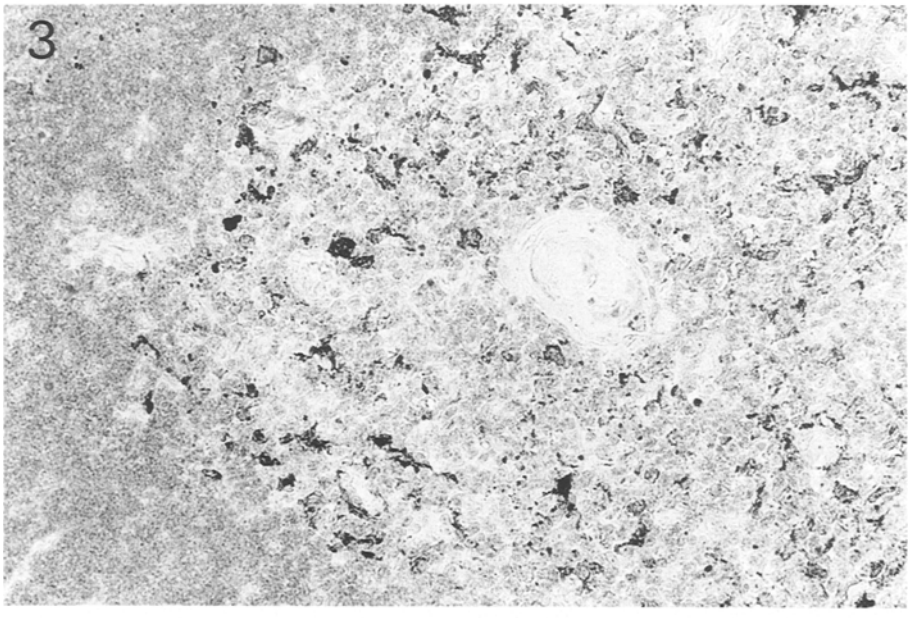


Fig. 3. Immunohistochemical staining of the thymus for ADF at 23 weeks of gestation shows positive staining of epithelio-reticular cells. The cortex and Hassall's corpuscles are negative for ADF. ×200

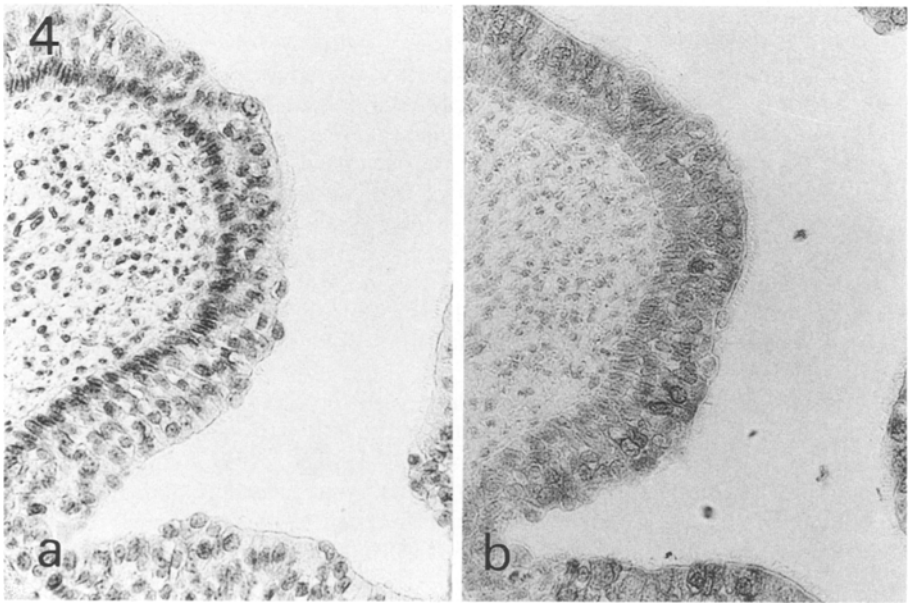


Fig. 4. Immunohistochemical staining of the oesophagus for ADF at 12 weeks of gestation: **a** negative control; **b** positive staining in the epithelium of the oesophagus, particularly in ciliated cells. ×200

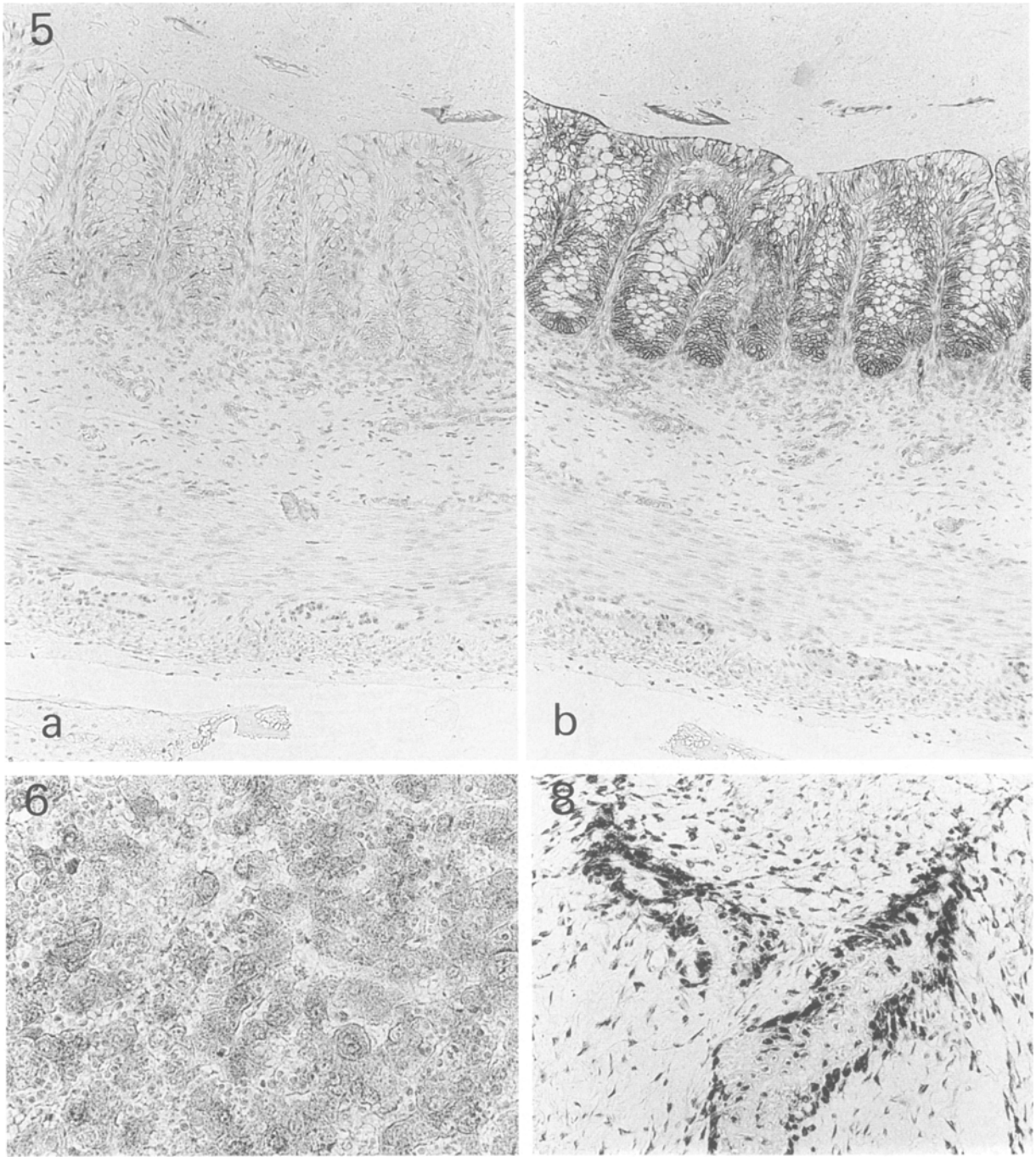


Fig. 5. Immunohistochemical staining of the intestine for ADF at 23 weeks of gestation: **a** negative control; **b** positive staining in the epithelium of the intestine. ×100

Fig. 6. Immunohistochemical staining of the liver for ADF at 23 weeks of gestation shows positive staining of hepatocytes. ×200

Fig. 8. Immunohistochemical staining of bone for ADF at 14 weeks of gestation shows positive staining of osteoblasts. ×100

and/or transformation during the adult life, such as steroid-producing cells of the ovary and decidual cells in the endometrium. In the primordial follicles of the adult ovary, neither follicular cells nor oocytes showed specific staining for ADF. According to the development of follicles, however, both granulosa cells and theca interna cells showed positivity for ADF. Moreover, ADF staining was observed in the corpus luteum. In the endometrium, the stromal cells transformed into the pre-decidual cells during the luteal phase and decidual cells during pregnancy showed strong positivity for ADF (Table 1).

Discussion

Thioredoxin was originally described as a co-enzyme involved in the reduction of ribonucleotides to deoxyri-

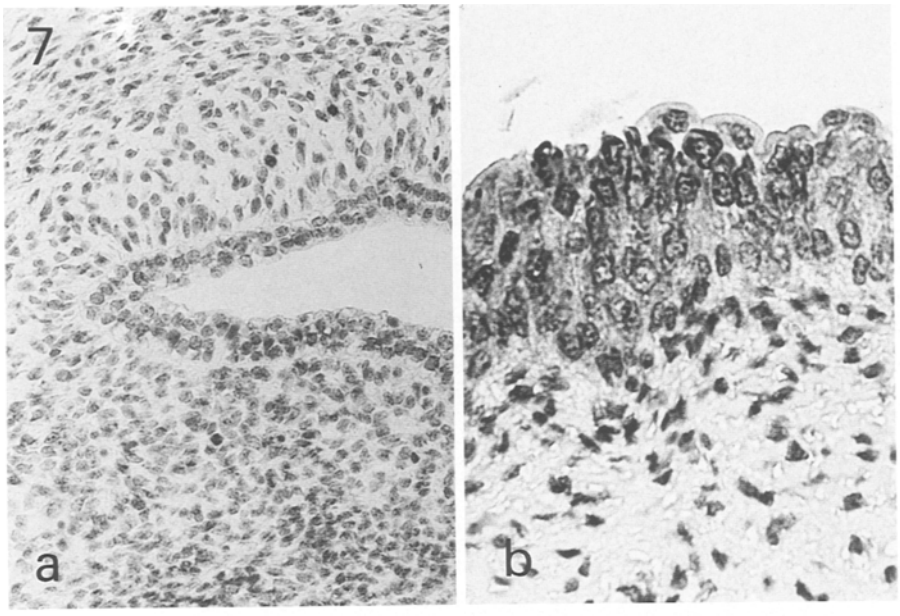


Fig. 7. Immunohistochemical staining of the urogenital for ADF is negative at 8 weeks of gestation (**a**), while the bladder epithelium is positive at 20 weeks of gestation (**b**). **a** ×200, **b** ×400

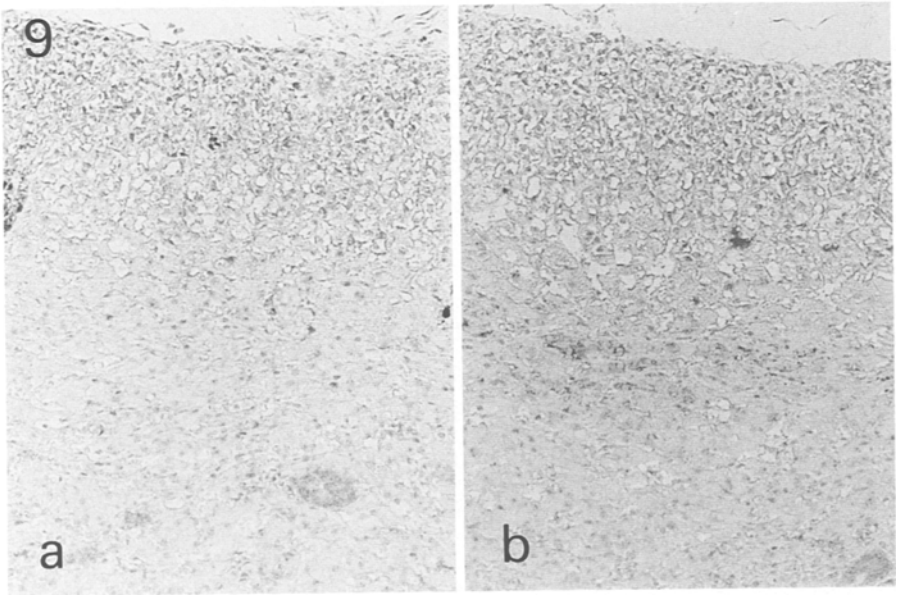


Fig. 9. Immunohistochemical staining of the adrenal gland for ADF at 13 weeks of gestation: **a** negative control; **b** positive staining of the inner zone of the cortex. ×100

bonucleotides (Laurent et al. 1964). Holmgren (1985) studied the function of thioredoxin in various species of both plants and animals. Thioredoxin contains a redox-active disulphide (−Cys−Gly−Pro−Cys−) and has a variety of biological activities as a hydrogen donor. It serves as an intracellular protein component in eukaryotic cells and is involved in many biologically important reactions (Holmgren 1985). Although originally isolated from the supernatant of HTLV-1-transformed cells as a cytokine-like factor, homology comparison using a protein data base revealed a similarity between ADF and thioredoxin (Tagaya et al. 1989). The dithiol-reducing activity of ADF was examined and it was shown to have a potent insulin-reducing activity (Tagaya et al. 1989, 1990). Furthermore, the purified human thioredoxin provided by Holmgren strongly reacted with anti-ADF antibodies in Western blotting, suggesting that ADF is a human counterpart of thioredoxins (unpublished observation).

The current study describes the immunohistochemical localization of ADF in human fetal tissues using an antibody against the C-terminal peptides of the ADF protein. ADF was found to be widely distributed in different organs and tissues during the fetal period. The anti-ADF antibody reacted with a large number of endoderm-derived tissues, such as the medullary cells of the thymus, lung eptihelium, the epithelium of the digestive tract, hepatocytes, and the bladder epithelium. However, among the ectoderm-derived tissues, only peripheral nerve cells, hair follicles, and sebaceous gland cells showed reactivity with the anti-ADF antibody. Staining was also selective in the mesoderm-derived tissues. For example, bone and cartilage were negative for ADF, except that osteoblasts showed moderate reactivity. The dendritic cells in the spleen and lymph nodes showed positivity for ADF. Moreover, the proximal tubules of the kidney, the cells which were destined to differentiate into ciliated cells in the fallopian tube and in the efferent

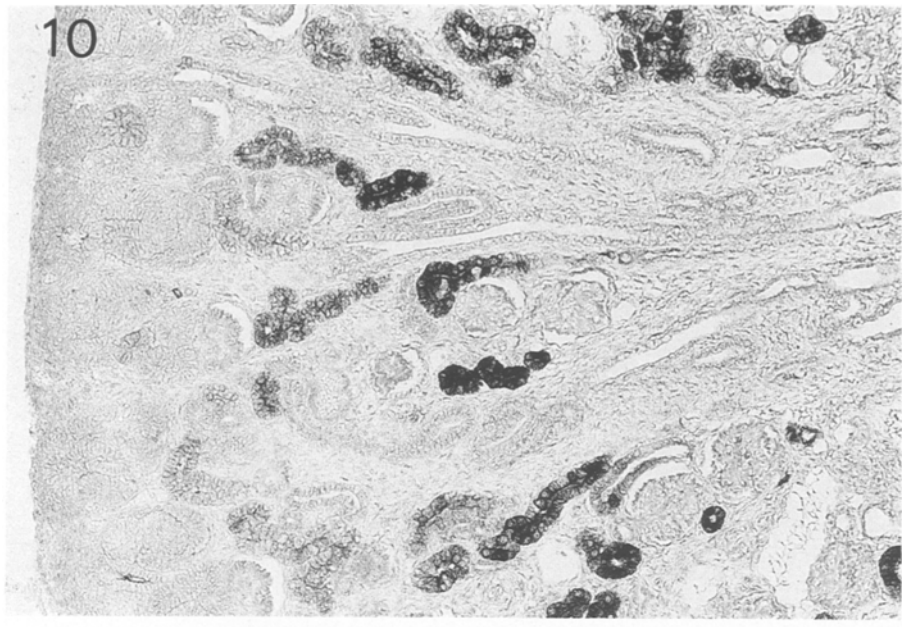


Fig. 10. Immunohistochemical staining of the kidney for ADF at 23 weeks of gestation shows positive staining of the proximal tubules. The glomerulus is negative for ADF. ×100

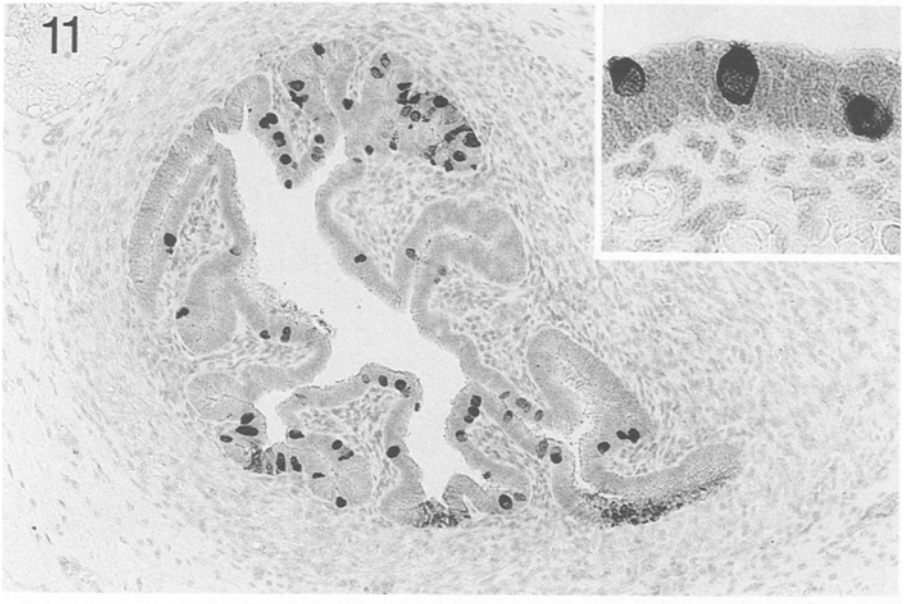


Fig. 11. Immunohistochemical staining of the fallopian tube for ADF at 23 weeks of gestation shows positive staining of the tube epithelium. The cells which are destined to differentiate into ciliated cells are selectively positive for ADF, ×100. The *inset* shows a higher magnification of ADF-positive cells. ×400

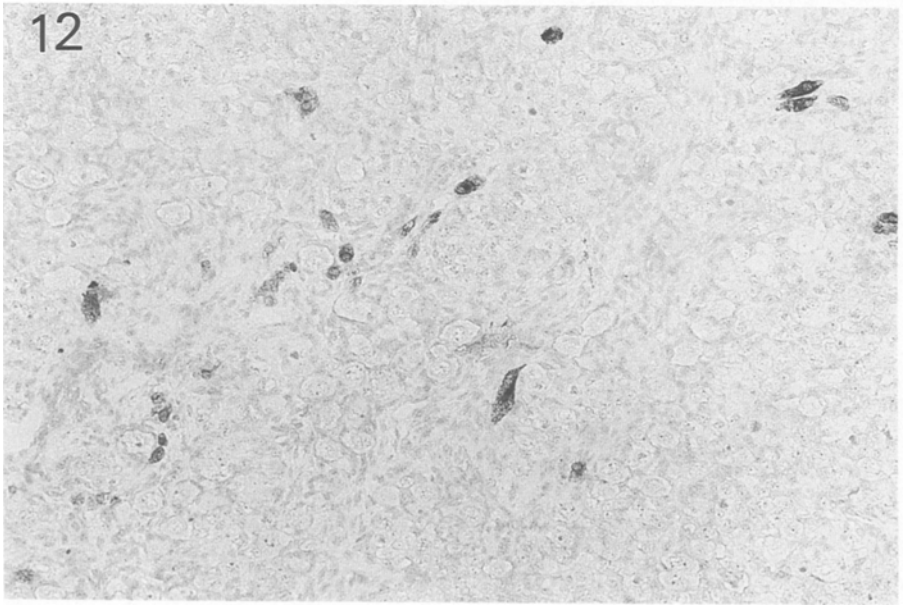
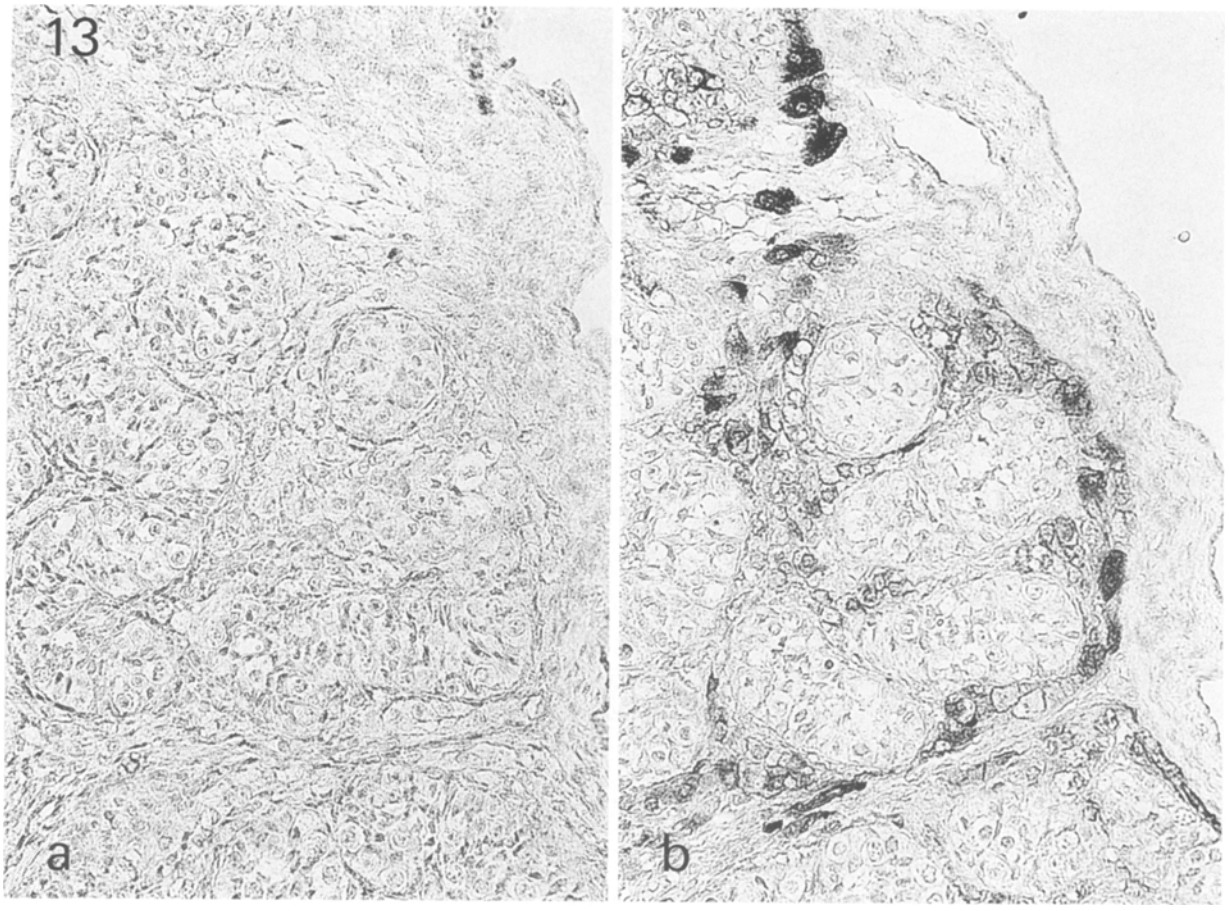


Fig. 12. Immunohistochemical staining of the ovary for ADF at 23 weeks of gestation shows positive staining of the interstitial cells. ×100



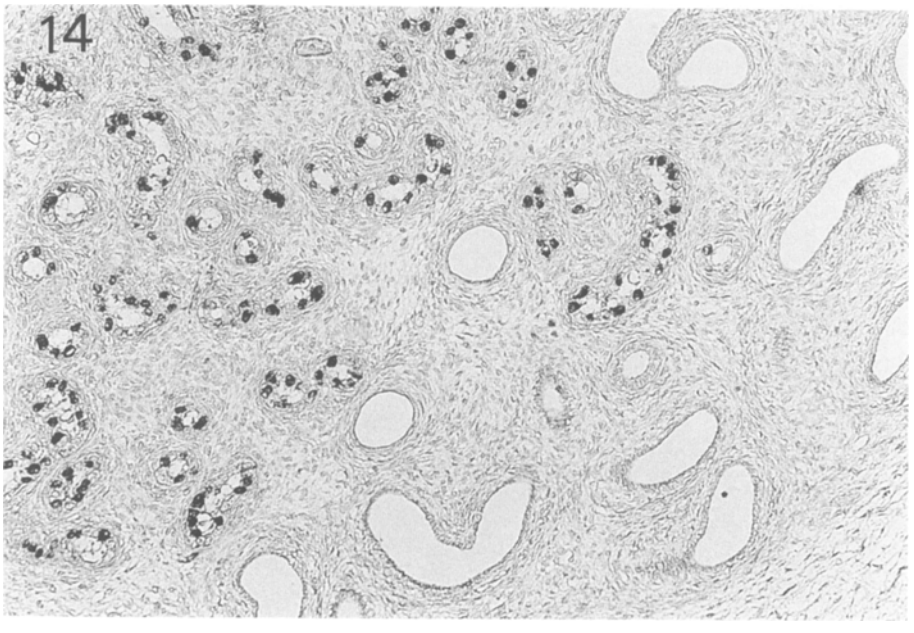


Fig. 13. Immunohistochemical staining of the testis for ADF at 23 weeks of gestation: **a** negative control; **b** positive staining of Leydig cells. ×200

Fig. 14. Immunohistochemical staining of the testis for ADF at 23 weeks of gestation shows positive staining of the epithelium of the efferent ductules. The cells which are destined to differentiate into ciliated cells are selectively positive for ADF. ×100

ductules of the testis, the interstitial cells in the ovary, and the Leydig cells of the testis were all stained selectively and showed strong reactivity with the anti-ADF antibody. All other tissues observed in this study had little or no immunoreactivity. ADF was localized in either the nucleus or the cytoplasm of cells.

The immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats has been reported previously (Rozell et al. 1985). Thioredoxin localizes in hepatocytes, the epithelio-reticular cells of the thymus, the respiratory epithelium, the digestive tract epithelium, the adrenal cortex, the Leydig cells in the testis, and several other tissues in adult rats (Rozell et al. 1985). the localization of ADF in human fetal tissues

resembles that of thioredoxin in adult rat tissues. However, a few tissues, such as the brain, skin, and kidney, showed a different staining for ADF in humans and thioredoxin in the rat. The brain and keratinized cells of the epidermis have been reported to show reactivity for thioredoxin in the rat (Rozell et al. 1985), but both these tissues were negative for ADF in the human fetus. In contrast, the renal tubular cells were reported to show weak reactivity for thioredoxin in the rat (Rozell et al. 1985), but the proximal tubules for the fetal kidney showed strong reactivity for ADF. These staining differences may be partly due to differences of the antibodies used, species differences, and/or the immaturity of the human fetal tissues.

In general, it has been reported that growing or proliferating cells contain thioredoxin at some level and that variations exist in the immunoreactive thioredoxin content of non-proliferating, differentiated cells in relation to their metabolic activity, accumulation of cell products, and secretory activity (Holmgren 1985; Rozell et al. 1985). In the human fetus, endoderm-derived tissues showed a difference in staining according to the degree of differentiation of the epithelium. The epithelium of the urogenital sinus at 9 weeks of gestation was negative for ADF, whereas the digestive tract epithelium was positive at this stage. In addition, the bladder epithelium became positive for ADF by 20 weeks of gestation. Similarly, in the epithelium of the müllerian ducts, only the ciliated cells which differentiate by 20 weeks of gestation (Konishi et al. 1987) showed positivity for ADF. Thus, some cellular function(s) acquired by differentiation during the fetal period seem to be necessary for the expression of ADF. Among the hormone-producing cells in the human fetus, steroid-producing cells in the adrenal cortex, the ovary (interstitial cells) (Konishi et al. 1986) and the testis (Leydig cells), all of which are functionally active during the fetal period, showed strong reactivity for ADF. However, the peptide hormone-producing cells, which are less active during the early fetal period, seemed to be weakly positive for ADF. In addition, absorbtive cells in the digestive tract and the proximal tubules of the kidney had a tendency to express more ADF than secretory cells. Moreover, the ciliated cells in the fallopian tube, the oesophagus, and the efferent ductules of the testis were strongly positive for ADF. Therefore, those cells with functions requiring high energy levels, such as absorbing cells and ciliated cells, seemed to contain a large amount of ADF.

Almost all tissues reacted with the anti-ADF antibody during the early fetal period also showed positivity for ADF in the adult. Therefore, ADF was also found to be widely but selectively distributed in different organs and tissues during adult life. However, the intensity of staining in these tissues was weaker than that of the fetal period. The difference of staining intensity between the cells of fetus and adult probably corresponds not only to the cellular activity but also to the degree of differentiation of the cells. In addition, the observation of ADF staining in the tissues of female genital system which show the development and/or transformation during adult life, such as steroid-producing cells of the ovary and decidual cells in the endometrium, also suggests that ADF expression correlates with the cellular function of certain tissues.

The expression of ADF in the thymus was peculiar, since only the epithelio-reticular cells in the medulla were strongly positive for ADF. Considering that the function of ADF as a cytokine is IL-2 receptor induction (Teshigawara et al. 1985; Tagaya et al. 1988, 1990), ADF in the thymus may be involved in T-cell differentiation during the fetal period.

In conclusion, the thioredoxin systems in mammalian cells and particularly in humans are still largely uncharacterized. Our immunohistochemical study of the distribution of ADF, a thioredoxin homologue, in human fetal tissues provided some new information on thioredoxin systems in human cells. Thioredoxin may possess various specialized functions related to the formation of protein disulphides, secretory processes, or the regulation of various enzyme activities, and may even act as a messenger for hormonal and receptor mechanisms (Holmgren 1985). In the investigation of these postulated functions of thioredoxin, the study of ADF, which has a potent reducing activity as well as a cytokine-like activity, should give us some new insights into the mechanism of cell activation. Work is now in progress to analyse the mechanism of ADF-related activation of cells.

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