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Elevated concentrations of the β-subunit of S100 protein in renal cell tumors in rats

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Abstract Concentrations of α and β-subunits of S100 protein (S100-α and S100-β) in rat kidney neoplasms, including renal cell and mesenchymal tumors, were determined using a highly sensitive enzyme immunoassay, and both types immunohistochemically localized in tissue sections. Concentrations of S100-α in each histological type of rat tumor were lower than in normal kidney, whereas levels of S100-β (mean ± SE: 29.7 ± 14.2 ng/mg protein, n = 15) in renal cell tumors were significantly higher than in normal kidneys (0.55 ± 0.06 ng/mg protein, n = 7), or mesenchymal tumors (1.21 ± 0.43 ng/mg protein, n = 9). In normal rat kidney tissues S100-α was immunohistochemically positive in epithelial cells of the distal tubules, the thin limbs of loops of Henle, and the collecting ducts. No appreciable immunostaining for S100-β was found in any nephron segment. Both S100-α and S100-β were positive for renal cell tumors, indicating new appearance of the latter during renal carcinogenesis in rats.

Key words S100 protein · Rat · Carcinogenesis · Renal neoplasms

S100 protein is a dimeric, acidic protein with a molecular mass of 21 kDa, belonging to the group of the EF-hand type calcium-binding proteins including troponin C, calmodulin, and parvalbumin. It is composed of two distinct subunits: the α-subunit (S100-α) and the β-subunit (S100-β). At least three forms of $S100a_0$, S100a, and S100b have been identified with subunit compositions of αα,αβ, and ββ, respectively [9, 10, 20]. S100 protein, previously considered specific to the nervous system, has now been found in a variety of non-nervous tissues, including heart muscles, kidney, thyroid glands and striated muscles [6, 12], as well as in neoplasms [7, 25]. The exact biological function of S100 protein remains unknown.

We earlier determined concentrations of S100-α and S100-β in human kidney and renal cell carcinoma (RCC) tissues, and immunohistochemically localized these subunits [25]. In evaluating levels of serum S100-α in RCC patients we further demonstrated that serum S100-α is a useful biomarker for monitoring clinical course [13, 26]. Semba et al. [23] have determined concentrations of S100-α and S100-β in the normal rat kidney, but no evaluations have been made on concentrations of S100-α and S100-β in chemical-induced renal tumors in rats.

In the present study we therefore first determined tissue concentrations of S100-α and S100-β in two types of chemical-induced renal tumors in rats and then investigated localization of both forms at the cellular level using an immunohistochemical approach.

Materials and methods

Animals and treatment

N-Ethyl-*N*-hydroxyethylnitrosamine (EHEN, Sakai Laboratories, Fukui, Japan) was used for induction of renal cell tumors by the method of Tsuda et al. [27] with some modification. In brief, female Sprague-Dawley rats (Charles River Japan, Atsugi, Japan) aged 6 weeks at the beginning of the experiment were maintained on a basal diet (Oriental M, Oriental Yeast, Tokyo) and tap water *ad libitum*. They were initially given 0.05% EHEN dissolved in their drinking water for 3 weeks, returned to normal tap water for the subsequent 49 weeks, and then put to death at the end of week 52.

N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN, Nakarai Chemicals, Kyoto, Japan) and uracil were used for the induction of mesenchymal tumors of the kidney as described by Nakano [21]. Female Sprague-Dawley rats (Charles River Japan, Atsugi, Japan) aged 5 weeks at the beginning of the experiment were maintained with free access to the basal diet and tap water. They were initially given 0.05% BBN dissolved in drinking water and 3% uracil in the basal diet simultaneously for 8 weeks, then returned to normal water and the basal diet, and put to death 52 weeks after the initial treatment.

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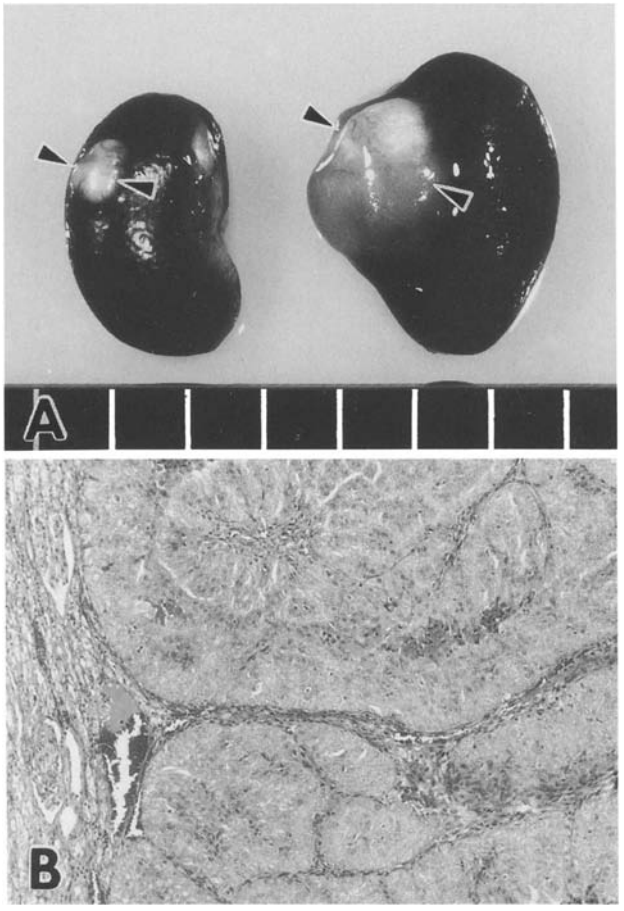

Fig. 1 **A** Gross appearance of *N*-ethyl-*N*-hydroxyethylnitrosamine (*EHEN*)-induced renal cell tumors in a rat. *Arrowheads* indicate bilateral tumors. **B** Microscopic appearance of the left tumor. H&E, ×80

In both experiments the kidneys were immediately excised for quantitative and immunohistochemical examination. A group of control rats not receiving any chemicals was also included.

Tissue samples

Tumor and control tissues were obtained from the above rats. Histological examination revealed that the 15 tumors from rats treated with EHEN were all renal cell tumors (Fig. 1) and that those in the kidneys of the rats treated with BBN and uracil were 9 mesenchymal tumors [21]. Normal kidney tissues from seven untreated rats were also prepared. For immunochemistry tissues were promptly frozen and kept at −80°C until analysis when they were homogenized at 0°C with 10 volumes (V/W) of 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgSO_4$. Homogenates were centrifuged at 4°C at 20 000 *g* for 20 min, and the soluble fractions used for the analysis.

For histological examination and immunohistochemistry, tissues from five cases of normal kidney and eight cases of renal cell tumors were fixed in 10% phosphate-buffered formalin for 24 h, processed routinely and embedded in paraffin. Four-micrometer-thick paraffin sections were stained with hematoxylin-eosin for histological diagnosis.

Antigens and antibodies

$S100a_0$ was purified from human pectoral muscles as described previously [14] and S100b from bovine brain [16]. Antibodies to S100-α and S100-β were raised in New Zealand rabbits by injecting the respective purified antigen with Freund's complete adjuvant, as described elsewhere [14, 16]. Antibodies monospecific to the two subunits were purified by immunoaffinity column chromatography using antigen-coupled Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The specificities of the purified antibodies thus obtained were reported previously [14, 16]. As secondary antibodies for immunohistochemistry, horseradish peroxidase (HRP)-labeled goat IgG Fab' fragments against rabbit IgG were prepared [25].

Immunoassay methods

Concentrations of S100-α and S100-β in the soluble fractions of tissues were determined by the sandwich-type enzyme immunoassay system developed by Kato et al. [14, 16]. In brief, extracts were incubated with polystyrene balls bearing immobilized monospecific rabbit antibodies to the respective subunits of S100 potein, and then the balls were incubated with the same antibodies labeled with β-D-galactosidase from *Escherichia coli*. The bound galactosidase activity was assayed with 4-methylumbelliferyl-β-D-galactoside as a substrate. Purified human S100-α and S100-β were, respectively, used as standards, and the results were expressed as human $S100a_0$ (αα) or bovine S100b (ββ) equivalent nanograms per milligram of soluble protein. Each assay system was highly sensitive, the limit of detection for both S100-α and S100-β being one picogram per test tube.

Immunohistochemistry

The indirect peroxidase-labeled antibody method was employed for the immunostaining as described previously [25]. In brief, 4-μm-thick paraffin sections were treated with 100% methanol and 0.3% hydrogen peroxide solution for 30 min to inactivate endogenous peroxidase. They were washed in phosphate-buffered saline (PBS), and then incubated with purified anti-S100-α IgG or anti-S100-β IgG for 60 min at room temperature. For control sections, antibodies absorbed with the purified respective antigen were substituted for the primary antibodies. After being washed in PBS, the sections were incubated with HRP-labeled secondary antibodies for 60 min at room temperature. After further washing in PBS, they were reacted with 0.025% 3,3'-diaminobenzidine solution containing 10 mM hydrogen peroxide and 10 mM sodium azide, and then counterstained with methyl green.

Other methods

Protein concentrations of the tissue extracts were determined with the aid of a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, Calif., USA), which utilizes the principle of protein-dye binding [3]. Quantitative data were expressed as mean ± standard error (SE) values and the results compared using the Mann-Whitney *U*-test.

Results

Concentrations of S100-α and S100-β in tissues of normal rat kidneys and renal tumors

Figures 2 and 3 illustrate the distributions of data for concentrations of S100-α and S100-β, respectively, in normal kidneys, renal cell tumors, and renal mesenchymal

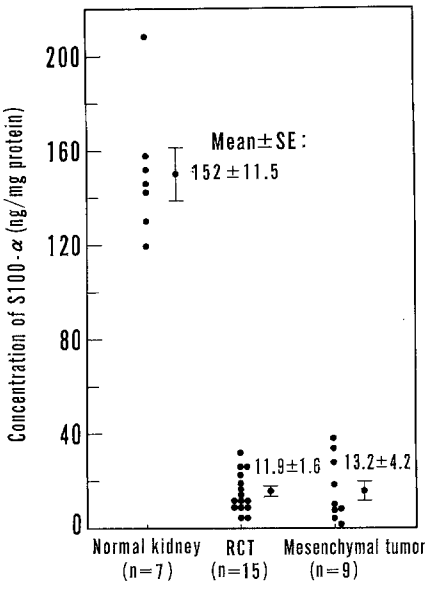

Fig. 2 Concentrations of S100-α in tissues of normal kidneys, renal cell tumors (*RCT*), and renal mesenchymal tumors. Levels of S100-α in each histological type of tumor were significantly lower than in normal kidneys ($P < 0.001$)

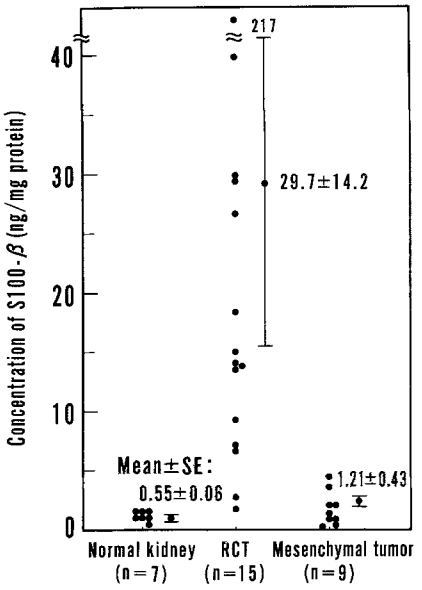

Fig. 3 Concentrations of S100-β in tissues of normal kidneys, renal cell tumors (*RCT*), and renal mesenchymal tumors. Levels of S100-β were significantly higher in RCTs than in normal kidneys or mesenchymal tumors ($P < 0.001$)

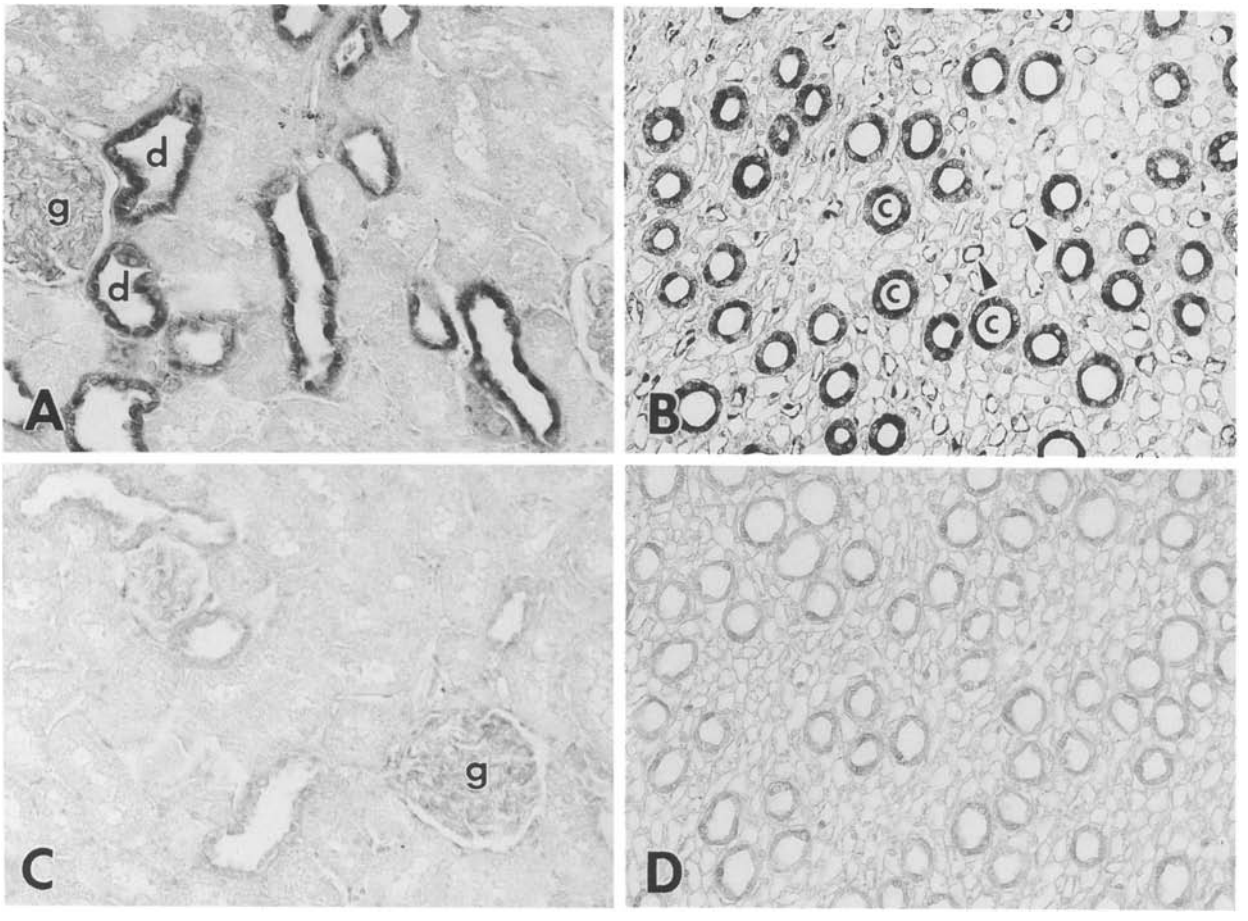

Fig. 4A–D Immunohistochemical localization of S100-α and S100-β in tissues of rat normal renal cortex (**A**, **C**) and medulla (**B**, **D**). **A**, **B** S100-α is localized in the cytoplasm and occasionally in the nucleus of epithelial cells of distal tubules (*d*) in the cortex, and thin limbs of loops of Henle (*arrowheads*) and collecting ducts (*c*) in the medulla. **C**, **D** S100-β is negative in renal tubules. *g*, Glomerulus. Indirect immunoperoxidase method, ×165

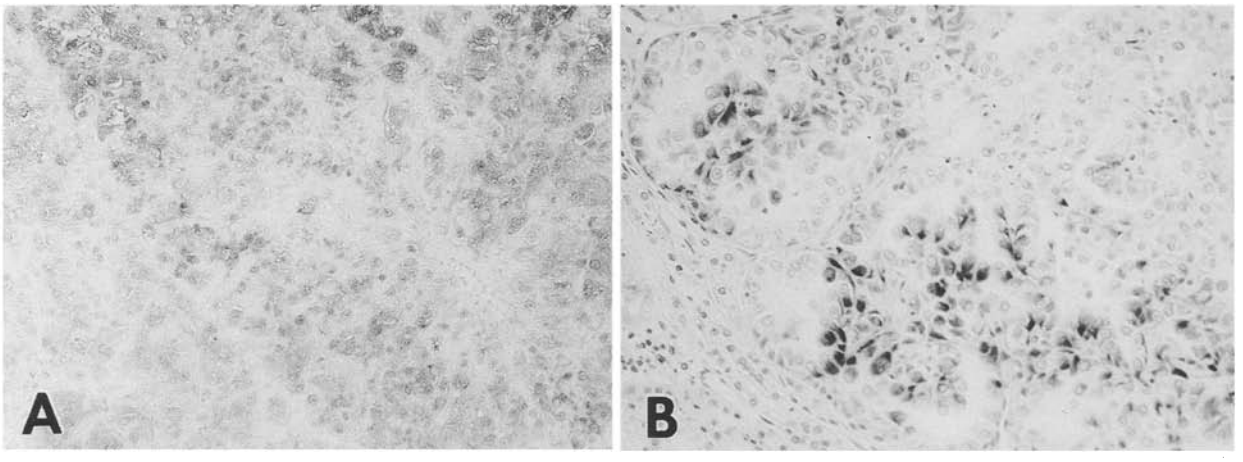

Fig. 5A, B Immunohistochemical localization of S100-α (A) and S100-β (B) in tissues of a rat renal cell tumor. Both S100-α and S100-β are localized in the cytoplasm and occasionally in the nucleus of tumor cells. Indirect immunoperoxidase method, × 165

tumors. Concentrations of S100-α in each histological type of tumor were significantly lower than in normal kidneys ($P < 0.001$), whereas levels of S100-β in renal cell tumors were significantly higher than in normal kidneys or mesenchymal tumors ($P < 0.001$).

Immunohistochemical localization of S100-α and S100-β in tissues of normal rat kidneys and renal cell tumors

Figure 4 illustrates the immunohistochemical localization of S100-α and S100-β in the cortex and medulla of a rat normal kidney. S100-α was found to be immunohistochemically positive in the cytoplasm and occasionally the nucleus of epithelial cells of the distal tubules (Fig. 4A), the thin limbs of loops of Henle, and the collecting ducts (Fig. 4B). No appreciable immunostaining for S100-β was found for the nephron segment (Figs. 4C and 4D).

Of eight cases of renal cell tumor tissue studied, all stained positively for S100-α and six (75%) for S100-β. Figure 5 illustrates the immunohistochemical localization of S100-α and S100-β in a typical renal cell tumor. In both cases localization was in the cytoplasm and occasionally in the nuclei of the tumor cells. The intensity of staining varied from case to case, and from site to site within individual tumors. No relationship between the staining for S100-α or S100-β and the grade of tumor differentiation was found. Control sections of tumor tissues treated with antibodies pre-absorbed with the respective antigens were uniformly negative.

Discussion

The present immunochemical and immunohistochemical study of S100-α and S100-β in normal renal tissues and renal tumors of rats revealed decreased concentrations of the former and increased concentrations of the latter subunit in renal cell tumors. In rat tissues S100-α was positively stained in the epithelial cells of distal tubules, thin limbs of loops of Henle, and collecting ducts in line with the finding reported earlier by Molin et al. [19]. In human renal tissues we found S100-α to be positive in proximal tubules, thin limbs of Henle and collecting ducts [25]. Whether this different localization of S100-α between rat and human renal tissues reflects functional variation remains to be investigated further. The fact that S100-β was scarcely detected in tissues of rat normal kidney (mean concentration of 0.55 ng/mg protein) is broadly in line with the low levels we found in human kidneys (1.96 ng/mg protein) [25]. The highly sensitive enzyme immunoassay enabled us to detect small amounts of S100-β. The present study revealed that no immunostaining for S100-β was found in any nephron segment, again in confirmation of the results of Molin et al. [19].

Previous histochemical and electron microscopic studies have revealed that human renal cell carcinomas are generally derived from proximal renal tubules [2]. Several investigators have disclosed that the majority of rat renal cell tumors induced by chemical agents also arise from proximal tubules and a minority from collecting ducts [1, 11, 18]. Because S100-β was not immunohistochemically positive in epithelial cells of normal renal tubules and because renal cell tumors in rats contained significant amounts of S100-β, the present study suggests that this subunit was newly expressed during carcinogenesis in the model used. However, further investigation using the in situ hybridization technique is needed to clarify whether the immunochemically and immunohistochemically detectable S100-β is really produced in the cells of renal cell tumors.

The biological significance of S100 protein remains unclear although several biological roles have been proposed in recent years. Regarding intracellular functions, the S100 protein modulates protein phosphorylation [22] and microtubule assembly [4], and activates a brain aldolase isozyme [28] and skeletal muscle adenylate cyclase [5]. Regarding extracellular roles, S100-β protein

secreted from glial cells stimulates neurite outgrowth in the developing brain [17]. S100 proteins are released from anterior pituitary cells [8] and adipocytes [15, 24]. In the kidney, Molin et al. [19] speculated that S100 protein might be associated with regulation of pH and electrolyte and water secretion/absorption. While functional differences between the two subunits in this organ remain unclear, the present findings do provide an important clue in that S100-β appears in rat renal cell tumors during their development.

References

1. Bannasch P, Zerban H (1990) Animal models and renal carcinogenesis. In: Eble JN (ed) Tumors and tumor-like conditions of the kidneys and ureters. Churchill Livingstone, New York, p 1
2. Bennington JL, Beckwith JB (1975) Renal adenocarcinoma. In: Firminger HI (ed) Tumors of the kidney, renal pelvis, and ureter, fascicle 12. Armed Forces Institute of Pathology, Washington DC, p 93
3. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248
4. Donato R, Isobe T, Okuyama T (1985) S-100 proteins and microtubules: analysis of the effects of rat brain S-100 (S-100b) and ox brain S-100a_0, S-100a and S-100b on microtubule assembly-disassembly. FEBS Lett 186:65
5. Fanò G, Fulle S, Torre GD, Giambanco I, Aisa MC, Donato R, Calissano P (1988) S-100b protein regulates the activity of skeletal muscle adenylate cyclase in vitro. FEBS Lett 240:177
6. Haimoto H, Hosoda S, Kato K (1987) Differential distribution of immunoreactive S100-α and S100-β proteins in normal nonnervous human tissues. Lab Invest 57:489
7. Ishiguro Y, Kato K, Ito T, Nagaya M (1983) Determination of three enolase isozymes and S-100 protein in various tumors in children. Cancer Res 43:6080
8. Ishikawa H, Nogami H, Shirasawa N (1983) Novel clonal strains from adult rat anterior pituitary producing S-100 protein. Nature 303:711
9. Isobe T, Okuyama T (1981) The amino-acid sequence of the α subunit in bovine brain S-100a protein. Eur J Biochem 116:79
10. Isobe T, Ishioka N, Masuda T, Takahashi Y, Ganno S, Okuyama T (1983) A rapid separation of S100 subunits by high performance liquid chromatography: the subunit compositions of S100 proteins. Biochem Int 6:419
11. Ito N (1973) Experimental studies on tumors of the urinary system of rats induced by chemical carcinogens. Acta Pathol Jpn 23:87
12. Kato K, Kimura S (1985) S100a_0 (αα) protein is mainly located in the heart and striated muscles. Biochim Biophys Acta 842:146
13. Kato K, Haimoto H, Ariyoshi Y, Horisawa M, Washida H, Kimura S (1985) High levels of S-100a_0 (αα) protein in tumor tissues and in sera of patients with renal cell carcinoma. Jpn J Cancer Res 76:856
14. Kato K, Haimoto H, Shimizu A, Tanaka J (1987) Enzyme immunoassay for measurement of the α subunit of S100 protein in human biological fluids. Biomed Res 8:119
15. Kato K, Suzuki F, Ogasawara N (1988) Induction of S100 protein in 3T3-L1 cells during differentiation to adipocytes and its liberating by lipolytic hormones. Eur J Biochem 177:461
16. Kimura S, Kato K, Semba R, Isobe T (1984) Regional distribution of S-100a_0 (αα), S-100a (αβ), and S-100b (ββ) in the bovine central nervous tissue determined with a sensitive enzyme immunoassay system. Neurochem Int 6:513
17. Kligman D, Marshak DR (1985) Purification and characterization of a neurite extension factor from bovine brain. Proc Natl Acad Sci USA 82:7136
18. McGiven AR, Ireton HJC (1972) Renal epithelial dysplasia and neoplasia in rats given dimethylnitrosamine. J Pathol 108:187
19. Molin S-O, Rosengren L, Baudier J, Hamberger A, Haglid K (1985) S-100 alpha-like immunoreactivity in tubules of rat kidney: a clue to the function of a "brain-specific" protein. J Histochem Cytochem 33:367
20. Moore BW (1965) A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun 19:739
21. Nakano Y (1991) Induction of renal pelvic tumors in rats by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) and uracil. Nagoya J Med Sci 53:65
22. Patel J, Marangos PJ, Heydorn WE, Chang G, Verma A, Jacobowitz D (1983) S-100-mediated inhibition of brain protein phosphorylation. J Neurochem 41:1040
23. Semba R, Kato K, Isobe T, Kashiwamata S (1987) Purification of S-100a_0 protein from rat kidney. Brain Res 401:9
24. Suzuki F, Kato K, Nakajima T (1984) Hormonal regulation of adipose S-100 protein release. J Neurochem 43:1336
25. Takashi M, Haimoto H, Murase T, Mitsuya H, Kato K (1988) An immunochemical and immunohistochemical study of S100 protein in renal cell carcinoma. Cancer 61:889
26. Takashi M, Zhu Y, Hasegawa S, Miyake K, Takagi Y, Kato K (1990) Evaluation of the alpha subunit of S100 protein in sera as a tumor marker for renal cell carcinoma. Jpn J Clin Urol 44:1055
27. Tsuda H, Sakata T, Masui T, Imaida K, Ito N (1984) Modifying effects of butylated hydroxyanisole, ethoxyquin and acetaminophen on induction of neoplastic lesions in rat liver and kidney initiated by *N*-ethyl-*N*-hydroxyethylnitrosamine. Carcinogenesis 5:525
28. Zimmer DB, Van Eldik LJ (1986) Identification of a molecular target for the calcium-modulated protein S100: fructose-1,6-bisphosphate aldolase. J Biol Chem 261:11424