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Expression of CD44 Isoforms Carrying Metastasis-associated Sequences in Newborn and Adult Rats

Karin Wirth, Robert Arch, Chezian Somasundaram, Martin Hofmann, Bernd Weber, Peter Herrlich, Siegfried Matzku and Margot Zöller

Expression of a splice variant of CD44, recognised by the monoclonal antibody (Mab) 1.1ASML, confers metastatic potential to non-metastasising tumour cells (*Cell* 1991, 65, 13–24). To explore whether the metastasis-associated variant of CD44 (CD44v) is expressed under physiological conditions, tissues of newborn and adult rats were stained with the Mab 1.1ASML. The 1.1ASML epitope is, indeed, expressed on the basal layer of the epidermis and the hair follicles as well as on cryptic epithelia in the gut. In addition, ductal epithelia of the pancreatic gland of newborn rats express CD44v. This pattern of expression differs from that of standard lymphocyte CD44 (CD44s). The anti-CD44s mAB Ox50 predominantly stains connective tissue. Although different variants of CD44 may express the epitope recognised by 1.1ASML, cells expressing CD44v share properties with metastasising tumour cells: the stage of proliferation and a restricted degree of mobility. Thus, during metastatic progression tumour cells may reactivate the expression of gene segments which serve highly specialised functions in embryonic and adult tissues.
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INTRODUCTION

THE POLYMORPHIC glycoprotein CD44 [1–4] has been originally described as a homing receptor of lymphocytes [5–7]. Apart from the involvement of CD44 in adhesion of lymphocytes to high endothelial venules, CD44 appears to be involved in lymphocyte maturation and activation [8–10]. Expression has also been found in non-haematopoetic tissues and in a variety of tumour cells [11–16]. While the standard form of CD44 (CD44s) is most abundant on haematopoetic cells, recent reports have shown that tumour cells frequently express larger isoforms of CD44, where additional exons are inserted in the membrane proximal extracellular domain [3, 13, 17–20]. The expression of one class of variants (CD44v), recognised by the Mab 1.1ASML, is causally involved in the formation of tumour metastases [17–19].

The explicit role of CD44v in metastasis formation prompted us to search for its expression in normal tissue. If CD44v was expressed under physiological conditions, the expression pattern, possibly, could provide clues as to the function of CD44v in metastasis formation. Here we show that the epitope for 1.1ASML is expressed in only a very restricted number of normal rat tissues suggesting one or several specialised functions of these CD44 variants.

Correspondence to M. Zöller at the Res. Program Diagnostics and Exp. Therapy, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 6900 Heidelberg 1, Germany.
K. Wirth, R. Arch, C. Somasundaram and B. Weber are at the Institut für Radiologie und Pathophysiologie, Deutsches Krebsforschungszentrum, Heidelberg; M. Hoffmann and P. Herrlich are at Kernforschungszentrum, Karlsruhe, Institut für Genetik und Toxikologie; P. Herrlich is also at the Institut für Genetik, Universität Karlsruhe; and S. Matzku is at Merck, Darmstadt, Germany.
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MATERIALS AND METHODS

Rats and tumours

BDX rats were obtained from Charles River, Sulzfeld, F.R.G. Rats were kept under specific pathogen-free conditions. In most experiments, animals were used at the age of 6 weeks. In addition, foetuses of around 10, 14, 18 and 21 days of gestation and litters of 24 h, 3, 7, 10, and 14 days and adult rats of 4, 26, 52, and > 52 weeks were analysed.

BSp73ASML is the metastasising subline of a pancreatic adenocarcinoma in the BDX rat strain [21]. The tumour line was maintained in culture using RPMI 1640 supplemented with antibiotics, L-glutamine and 10% foetal calf serum.

Antibodies

Mab 1.1ASML, previously designated A2.6, (IgG1) was derived from a fusion of Sp2/0 with BALB/c spleen cells after hyperimmunisation with BSp73ASML. It recognises exclusively the metastasising BSp73ASML subline [17, 22]. The target structure of Mab 1.1ASML on BSp73ASML cells is a splice variant of CD44 containing additional domains (exons v4 to v7 [10, 19]), that are not present on CD44s [17–19]. The epitope for Mab 1.1ASML is on exon v6. Thus, only cells carrying v6 sequences will be stained with Mab 1.1ASML. Culture supernatants of Mab 1.1ASML were purified by passage over a Protein A Sepharose 4B column, IgG1 being eluted with 0.1 mol/l citrate, pH 6.0. The Mab Ox49 (IgG2a) and Ox50 (IgG1) recognise the rat equivalent of CD44s/Pgp1 (A.F. Williams, University of Oxford, personal communication). Fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG1 and IgG2a were obtained from Southern Biotechnology Association.

Tissues and preparation

The following tissues were excised, mounted on cork plates, frozen in Isopentan (chilled in liquid nitrogen) and stored at −70°C: brain, lung, heart, submandibular gland, thyroid gland, liver, gut, kidney, pancreatic gland, ovary, uterus, omentum, muscle, skin and bone (newborn rats, only).

Determination of epitope density and proximity

Titrated numbers of tumour cells (4×10^5 cells per well, eight replicates) were dispensed into U-shaped microtitre plates, centrifuged and incubated with an excess of ^{125}I-labelled Mab

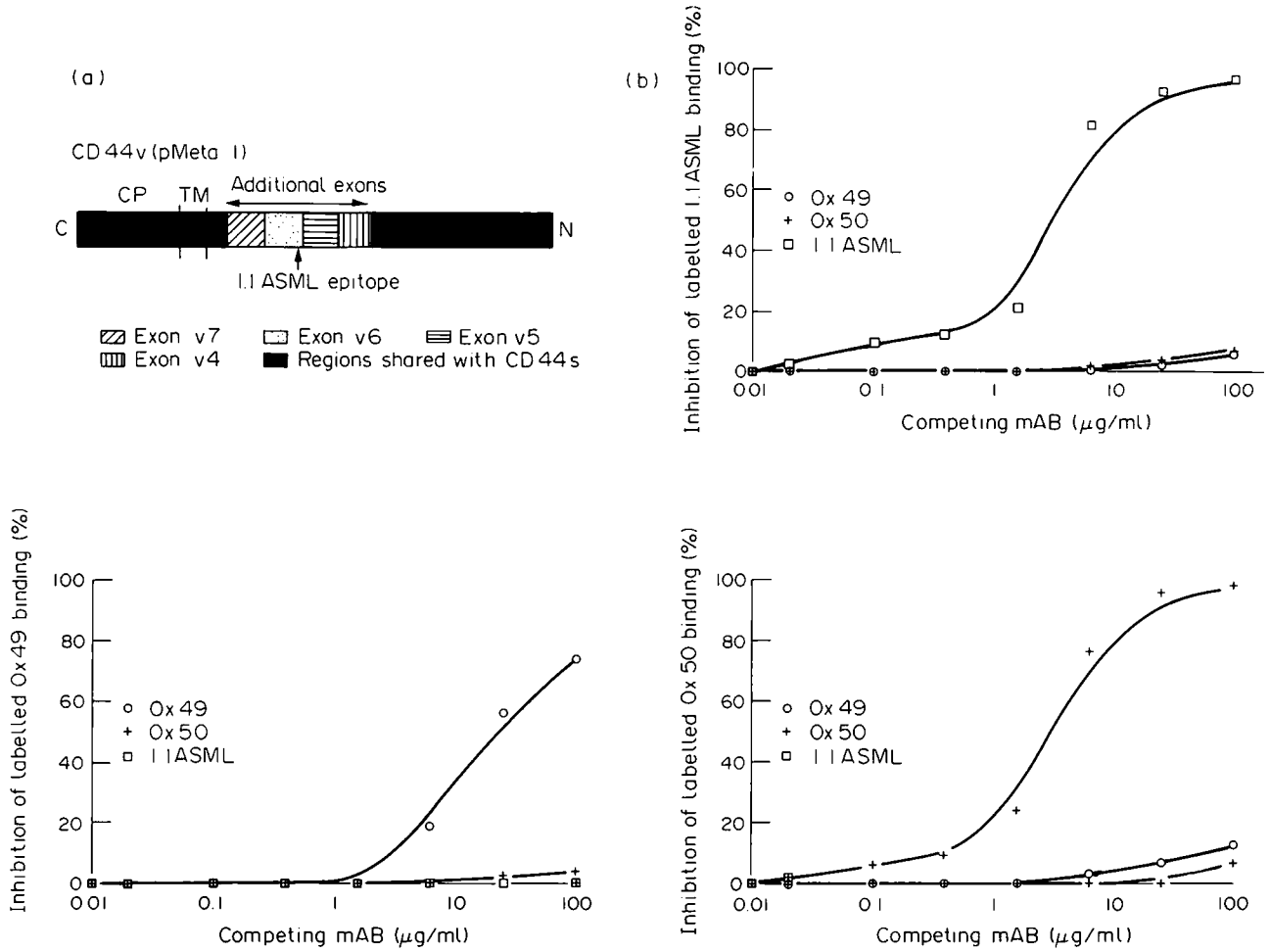

Fig. 1. The metastasis-associated CD44 variant and definition of anti-CD44s and anti-CD44v monoclonal antibodies. (a) Diagram of the metastasis-associated CD44 variant: the metastasis-associated CD44v contains four additional exons, v4 to v7, their position in CD44s being shown (CP and TM: cytoplasmic and transmembrane domains of CD44s). (b) Exclusion of competitive binding of Mab Ox49, Ox50 and 1.1ASML: BSp73AS-14 cells were incubated for 1 h with titrated amounts of Ox49, Ox50 or 1.1ASML (0–100 μg/ml). After washing, ^{125}I-labelled Ox49, Ox50 and 1.1ASML, respectively, was added, cells were incubated and washed again before cell-bound radioactivity was determined in a τ-counter. The percentage of inhibition of binding by unlabelled Mab is shown. Competitive binding studies with BSp73AS and BSp73ASML cells revealed similar results, except that Mab 1.1ASML did not bind to BSp73AS cells.

Ox49 or Ox50 or 1.1ASML. After 2 h at 0°C with continuous shaking, cells were washed and counted in a γ-counter. From the number of counts per tube the number of bound anti-CD44s and anti-CD44v was calculated and designated as the "apparent" epitope density, taking into account that one antibody molecule might have bound to one or two epitopes [23].

To evaluate whether epitopes may be in close proximity so that binding of anti-CD44s may interfere with binding of anti-CD44v and vice versa, tumour cells were preincubated with titrated amounts of unlabelled Mab at 4°C for 1h, plates were washed and ^{125}I-labelled Mab were added for an additional 2h at 4°C. Cells were kept in suspension by gentle shaking throughout the incubation periods. The percentage of inhibition of binding of ^{125}I-labelled Mab by unlabelled Mab is shown.

Staining of tissue sections

Frozen tissues were cut into 5 μm sections and were mounted onto slides, air-dried and incubated with the first antibody for 1 h at room temperature. Sections were washed with phosphate buffered saline (PBS) for 30 min and were incubated with FITC-labelled goat anti-mouse IgG1 or IgG2a antibodies at room temperature for 30 min. Stained sections were again washed in PBS for 2 h, were fixed with Entellan (Merck) and were analysed by fluorescence microscopy.

Whole body autoradiography

Whole body cryostat sections of rats were obtained 24 h after intravenous injection of 10 MBq of ^{125}I-labelled 1.1ASML. Processing and exposure of X-ray films was as previously described [24].

RESULTS AND DISCUSSION

Expression of CD44s and CD44v on metastatic tissue

For the analysis of expression of CD44s and of variant forms of CD44 carrying a metastasis-associated epitope (Fig. 1a), three Mab were used: Mab Ox49, Mab Ox50, both recognising epitopes on CD44s as detected on lymphoid cells, and 1.1ASML, which recognises an epitope on sequences encoded by exon v6 of the CD44 gene. Variants carrying this sequence were found to confer metastatic behaviour to non-metastasising tumour cells [17, 19].

To evaluate whether the epitopes for Ox40 or Ox50 may be absent or hidden on the metastasis-associated variant of CD44, epitope densities for Ox49, Ox50 and 1.1ASML were determined on BSp73AS-14 cells in comparison to BSp73AS and BSp73ASML cells. BSp73AS cells carry only CD44s, while BSp73AS-14 cells had been transfected with pMeta1, a construct containing full length cDNA of CD44v (exons v4–v7). Thus, in case epitopes of CD44s are present and accessible on the variant CD44 molecule, epitope density for Mab Ox49 and Ox50 should be increased on BSp73AS-14 cells in comparison to BSp73AS cells, in equimolar amounts compared to the density of epitopes for 1.1ASML. This was indeed the case as demonstrated for binding of Mab Ox50 and 1.1ASML (Table 1). Furthermore, binding of Ox49, Ox50 and 1.1ASML is not competitive (Fig. 1b), i.e. the possibility of stearic hindrance by bound Mab due to close proximity of the epitopes could be excluded. Thus, the metastasis-associated CD44v carries the epitopes of CD44s,

Table 1. Epitope density for Mab Ox50 and 1.1ASML on non-metastasising and metastasising tumour cells

Tumour	Epitope density for Ox50	1.1ASML
BSp73AS*	2.2×10^5	—
BSp73AS-14*	20.7×10^5	18.3×10^5
BSp73ASML*	18.3×10^5	16.3×10^5

*BSp73AS and BSp73ASML are the non-metastasising and metastasising sublines, respectively, of a rat pancreatic adenocarcinoma. BSp73AS-14 are BSp73AS cells transfected with pMeta1.

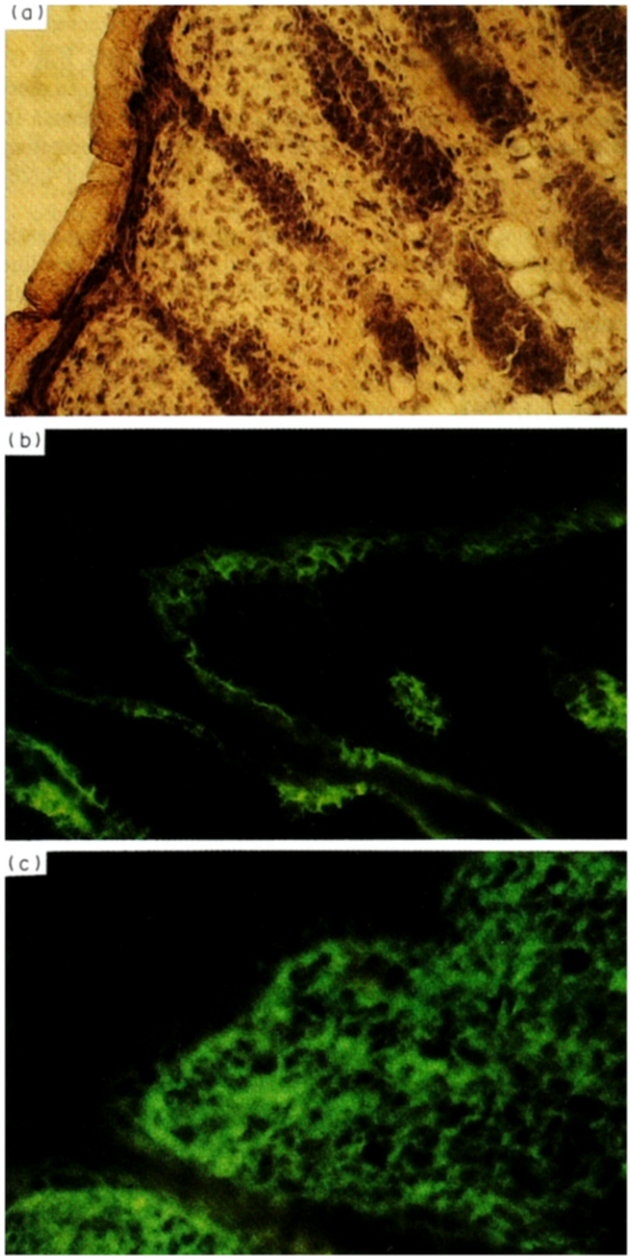

Fig. 2. Staining of newborn skin with 1.1ASML and Ox50. (a) Haematoxilin–eosin staining; (b) staining with 1.1ASML and fluorescein-labelled anti-mouse IgG1 antibodies; (c) staining with Ox50 and fluorescein-labelled anti-mouse IgG1. Ox50 stains connective tissue of the subcutis; 1.1ASML stains the basal layer of the epidermis including hair follicles.

which are recognised by Mab Ox49 and Ox50. All three Mabs recognise different epitopes on CD44s and CD44v, respectively.

Physiological expression of CD44v

The predominant feature of 1.1ASML staining is that there are only few cells, which are distinctly positive. We have described elsewhere that subsets of lymphoid cells express a variant of CD44 sharing the metastasis-associated epitope only during maturation and after antigenic stimulation [10]. Beyond that, only selected epithelial cells are stained with 1.1ASML.

In the skin, the basal layer of the epidermis including hair follicles are stained brightly with Mab 1.1ASML (Fig. 2). The suprabasal layer shows severely reduced staining, yet the epidermal cells do not bind the Mab Ox50 (and Ox49, not shown). This is surprising, because we have cloned CD44-related cDNA expressed in human keratinocytes and found that the human keratinocyte-specific CD44 variant carries all portions of CD44s [18]. Since the epitopes for Ox49 and Ox50 were accessible on the variant form of CD44 as expressed on tumour cells, the structure of the variant on keratinocytes must be differently modified or covered by another protein. Exclusive and bright staining with Mab Ox50 (and Ox49, data not shown) is, however, detected in the subcutis. CD44 expressed in the subcutis does obviously not contain or expose the 1.1ASML epitope.

Expression of CD44s in the subcutis and of a variant of CD44 in the basal layer of the epidermis is already observed at 14–15

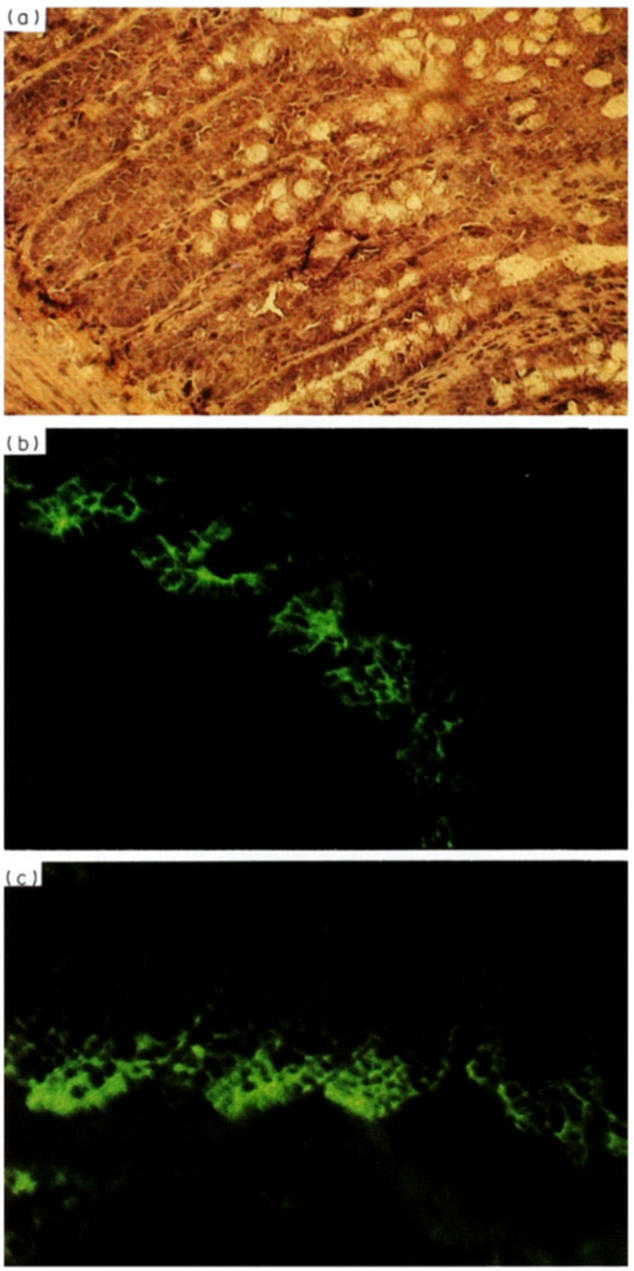

Fig. 3. Staining of the gut with 1.1ASML and Ox50. (a), (b) and (c) as described in Fig. 2. Cells in the base of the crypts stain brightly with Ox50 and 1.1ASML.

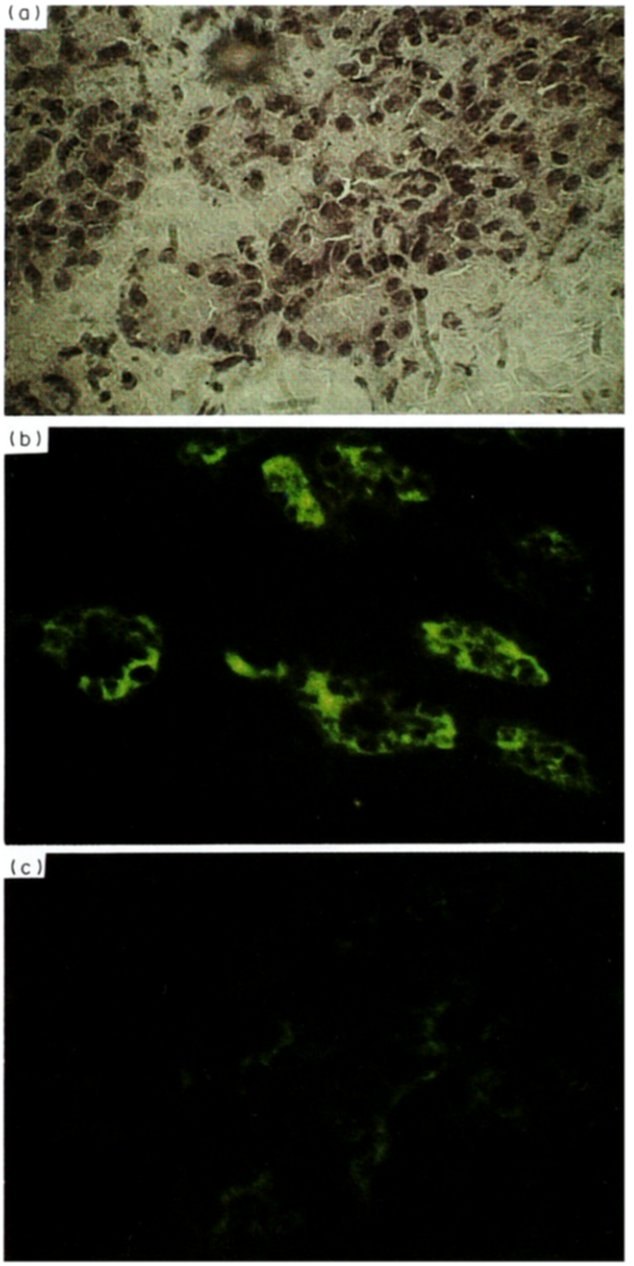

Fig. 4. Staining of the pancreatic gland with 1.1ASML and Ox50. (a), (b) and (c) as described in Fig. 2. In the pancreatic gland of newborn rats ductal epithelia stain with 1.1ASML, but not with Ox50, which faintly stains connective tissue. In the adult, neither 1.1ASML nor Ox50 stain pancreatic tissue (not shown).

days of gestation and remains stable throughout life (data not shown).

A different pattern of expression is found in the gut. Cells at the base of the crypts are stained brightly with Ox50, Ox49 (data not shown) and 1.1ASML (Fig. 3). This pattern of staining of cryptic cells is first observed at around 19 days of gestation and remains stable throughout life.

The only other examples of expression of the 1.1ASML-epitope were found exclusively in the prenatal (day 18–20 of gestation) and neonatal period, where the epitope is expressed in the submandibular and the thyroid gland (data not shown) and on ductal cells of the pancreatic gland, while islets, acini and connective tissue are negative (Fig. 4). Staining of the submandibular and the thyroid gland was no longer observed at birth and staining of ductal cells of the pancreatic gland diminished rapidly within the first week of postnatal life. Interestingly, glandular tissues and the ductal cells—like the basal layer of the epidermis—do not stain with Ox50 (and Ox49, data not shown). Since CD44-related cDNA of these tissues has not been cloned or examined by PCR it is not known whether the sequences are absent or shielded, yet the similarity to staining of keratinocytes is suggestive of the existence of a similar modification or conformation of a CD44 variant. In addition, the connective tissue of the glands is stained faintly with Ox50, as was also observed in the skin.

Faint staining with Ox49 and Ox50 is also seen in other connective tissues of newborn rats such as perimysium and kidney. Yet, neither in the prenatal stage nor during postnatal development nor in adult rats is any one of these tissues stained with 1.1ASML (data not shown).

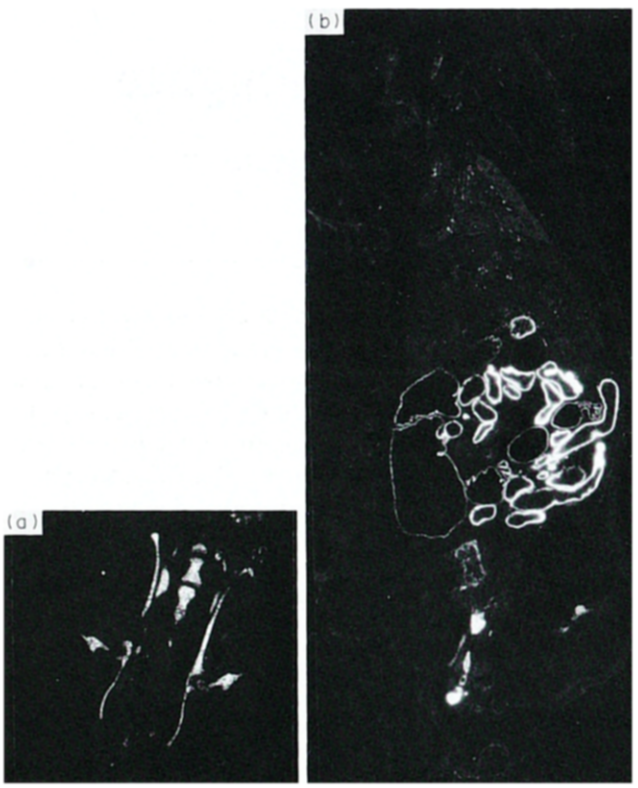

Fig. 5. Autoradiography with 1.1ASML. Adult rats received 10 MBq ^{125}I-labelled 1.1ASML, intravenously. Cryostat sections were performed afer 24 h. 1.1ASML localises predominantly (a) in the bone marrow (os sacrum, pelvic bone and femur are shown), and (b) in the gut.

Finally, it should be mentioned that accessibility of CD44v-positive cells *in vivo* varies significantly from that seen in tissue sections. After intravenous injection of ^{125}I-labelled 1.1ASML, cryostat sections of whole rats do not reveal any localisation of labelled 1.1ASML in the skin. Yet, bone marrow (Fig. 5a) and, especially prominently, intestinal tissue (Fig. 5b) is accessible for 1.1ASML.

Expression of CD44s and of variants thereof is quite abundant. It is not clear what properties they share, but the structure of CD44s and its variants suggests their role in cell–cell and cell–matrix interactions [12, 16, 25–30]. Expression of 1.1ASML-positive variants differs from variants described so far. Even taking into account that masking of the epitope for Mab 1.1ASML cannot be excluded and some sites of expression may have been missed,1.1ASML-positive variants are rare and, most strikingly, there is no example of permanent expression under physiological conditions. Since all forms of CD44 are transcribed from one promoter, expression of 1.1ASML-positive variants must be strictly regulated already on the level of splicing. The restricted pattern of transient expression suggests a very specialised function of 1.1ASML-positive variants. From our data we favour as a working hypothesis a role in directed locomotion/adhesion, which probably is accompanied by a temporary stimulus for proliferation.

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Tumour Necrosis Factor Increases Tumour Uptake of Co-administered Antibody–Carboxypeptidase G_2 Conjugate

R.G. Melton, J.A. Rowland, G.A. Pietersz, R.F. Sherwood and I.F.C. McKenzie

Increased tumour uptake of antibodies and antibody–drug conjugates has been demonstrated following pretreatment of animals with recombinant human tumour necrosis factor-α (rTNF-α) and interleukin 2 immunoconjugates. The experiments reported here were performed to determine whether improved tumour localisation of antibody–carboxypeptidase G_2 conjugates could be achieved, with a view to applying this technology to antibody-directed enzyme–prodrug therapy (ADEPT). B6CF$_1$ mice bearing the Ly-2.1$^+$ murine thymoma E3 were simultaneously injected with 2.0 μg rTNF-α and 3.5 μg (74 kBq) ^{125}I-labelled murine anti-Ly-2.1-CPG$_2$ conjugate. Mice in control groups received phosphate buffered saline in place of rTNF-α. The conjugate corresponded in molecular weight to a mixture of 1:1 and 2:1 (CPG$_2$:IgG) conjugate and retained its antigen binding specificity and enzymic activity *in vitro*. A significant increase in tumour uptake was observed 24 h after administration when rTNF-α-treated animals were compared to controls (28.1 ± 9.7% / g and 11.6 ± 2.3% / g, respectively). Other tissues, most notably gut, skin and kidney also showed an increased localisation of conjugate. By 48 h, analysis of tissue:blood ratios demonstrated that although tumour:blood ratios were significantly higher in rTNF-α-treated animals ($P < 0.05$), all the other tissue:blood ratios were not significantly different between the two groups.
Eur J Cancer, Vol. 29A, No. 8, pp. 1177–1183, 1993.

INTRODUCTION

ANTIBODY-DIRECTED enzyme–prodrug therapy (ADEPT) is a novel approach to cancer therapy in which antibodies directed against tumour-associated antigens are used as vectors for enzymes which are capable of converting inactive prodrugs to active drugs [1, 2]. Careful design of prodrug and selection of enzyme should make it possible to produce prodrugs which are not susceptible to activation by endogenous enzymes and are therefore non-toxic to the host, but which can be activated by enzyme, bound via antibody to the target tumour, to exert a