# Could Serum Antibody to Poly(ADP-Ribose) and/or Histone H1 Be Marker for Senile Dementia of Alzheimer Type?

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ABSTRACT: **Poly(ADP-ribosyl)ation has been focused on ischemic injury in the brain in relation to Alzheimer's disease (AD). We have measured IgG antibodies against poly adenosine diphosphate-ribose (pADPR) as well as histone H1 (H1) in 26 patients with either AD or with senile dementia of Alzheimer type (SDAT), and found that 80.7% (21/26) were positive for anti-pADPR IgG antibodies. Anti-H1 IgG antibodies were less positive (57.6%) (15/26) than anti-pADPR IgG antibodies, however, titers of both antibodies were well correlated ($r = 0.768$). Meanwhile, similar studies on 32 patients with systemic lupus erythematosus (SLE) who were positive for anti-pADPR antibody showed poor correlation ($r = 0.184$) and the difference in the correlation was statistically significant ($r < 0.01$). It is worthy of remark that anti-double-stranded (ds) DNA antibody, which is the hallmark of SLE, was negative in all dementia patients. Together with the findings that major subclass in dementia is both IgG1 and IgG2 and that in SLE was IgG2, the mode of production of anti-pADPR antibody in AD and SDAT is under different regulation mechanisms from that in SLE. Given the evidence that major target for ADP-ribosylation is H1 molecule, the association between anti-pADPR and anti-H1 in AD/SDAT makes sense and supports the concept that modification of proteins renders them immunogenic. Whatever the regulation is, parallel assay of two antibodies above would be of use not only for monitoring the disease process but also as a prodrome for possible subsets of SDAT and AD.**

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KEYWORDS: **poly(ADP-ribose); ADP-ribosylation; antipoly (ADP-ribose); Alzheimer's disease; blood–brain barrier (BBB); autoantigen**

## INTRODUCTION

Recent reports on patients with Alzheimer's disease (AD) that had a shift of histone H1 (H1) to the cell surfaces from nuclei in the astrocytes[1] as well as high levels of adenosine diphosphate (ADP) ribosylation reaction in the atrocytes[2] reminded us of our previous finding that immunization of rabbits with ADP-ribosylated histone enhanced the immune response not only to poly(ADP-ribose) (pADPR) but also to histone itself.[3] The presence of anti-pADPR antibody in patients with systemic lupus erythematosus (SLE) was also reported first by Kanai *et al.*[4] Dimerization of H1 by means of ADP-ribosylation reaction[5] is theoretically of value in the induction of autoimmunity in view of an established system of hapten ADP-ribose and carrier H1. Based on these findings, we measured the IgG immune responses to both H1 and pADPR in 20 patients with senile dementia of Alzheimer type (SDAT) and in 6 patients with AD; none had rheumatic diseases. Nevertheless, we have found that these patients respond to both antigens. The tight metabolic relationship between AD and pADPR and/or histone,[1,2] and immunological interrelationship between pADPR and histone[3] prompted us to study and compare the mode of immune responses to these antigens between patients with AD or SDAT, which are not affirmed as yet to be autoimmune diseases and those with SLE, a representative systemic autoimmune disease that shows high immune responses to nuclear antigens including histones and pADPR.[4,6] In these studies, a number of patients with AD and SDAT were small. Instead, we included serum samples from 59 elderly without AD or SDAT.

## MATERIALS AND METHODS

pADPR was synthesized from calf thymus nuclei in the presence of nicotinamide adenine dinucleotide and was purified as previously reported.[3] H1 was separated by cationic ion exchange chromatography (HiTrapSP; Amersham Biosciences, Uppsala, Sweden) from total histones purified from nuclei of HL-60 cells as previously reported.[7] Double-stranded (ds) DNA was isolated as the pure form of HL-60 cell nucleosomes, as described.[7] Purification of genomic DNA was done as well. pADPR thus purified was free from H1 and *vice versa*. Also, dsDNA was guaranteed to be free from nucleosomal proteins, as far as tested by agarose gel electrophoresis. Assay for IgG antibodies to each antigen was carried out by enzyme-linked immunosorbent assay (ELISA) in which antigens were directly coated to microtiter plates (Immulon 2HB, Chantilly, VA) under 25 mM Tris (pH 7.4) buffer containing

250 mM NaCl in order to eliminate the bias of precoating with glues, such as poly-L-lysine as described.[7] Diagnose of SDAT and AD were made according to DMS-IV (*Diagnostic and Statistical Manual of Mental Disorders*, 4th ed., American Psychiatric Association, Washington, D.C., 1994), and diagnosis for SLE was made according to *Updating the American College of Rheumatology Revised Criteria for the Classification of SLE (1997)*. Twenty-six patients with AD or SDAT who participated in this research were over 65 years old; whereas the age in average of 59 elderly participated as controls without particular diseases was 77 years. Moreover, the age in average of 40 patients with SLE was at least under 60 years. In order to obtain basal levels for anti-pADPR and anti-H1 antibodies, sera of 28 healthy persons without particular diseases under 60 years of age were used: their means plus 2 SD were adopted as a basal level of individual antibodies: the former was 0.173 A405 units and the latter was 0.105 A405 units. All sera collected from individual participants were stored at –70°C until use. It should be mentioned that this study was approved by the ethics committee of this hospital and bloods were drown under informed consent of subjects/patients or their guardians in cases of dementia patients.

## RESULTS

Six (100%) of 6 AD patients and 15 (70%) of 20 SDAT patients were positive for anti-pADPR antibodies (overall positivity of anti-pADPR antibody for patients with SDAT and AD was 80.7%, and 32 (80%) of 40 patients with SLE were positive for anti-pADPR antibodies. Also, 15 (57.6%) of 26 patients with SDAT or AD were positive for anti-H1 antibodies, whereas 12 (30%) of 40 patients with SLE were positive for anti-H1 antibodies. Profiles of autoantibodies in the two diseases positive for anti-pADPR antibodies are shown in FIGURES 1 and 2. The level of anti-pADPR antibodies in patients with SLE (FIG. 2) was higher than that in dementia (SDAT/AD) (FIG. 1) as was expected, however, the difference in the correlation coefficient ($r$) between two antibodies in an individual disease was statistically significant ($P < 0.01$); namely, the correlation coefficient ($r$) of 21 patients with dementia who were positive for anti-pADPR antibody was 0.768 (FIG. 1), while that of 32 SLE patients who were positive for anti-pADPR antibody was 0.184 (FIG. 2). It should be mentioned here that anti-dsDNA antibody, a hallmark of SLE, was 90% positive in patients with SLE as was expected, but it was totally negative in all patients with dementia (data not shown). To substantiate the relationship, we have further studied 59 cases of age-comparable (77 years old in average) elderly without either dementia or SLE and found that 37% (22/59) were positive for anti-pADPR IgG antibody but only 3.3% (2/59) were positive for anti-H1 IgG antibodies, none of 22 elderly positive for anti-pADPR being associated with anti-H1 at all (TABLE 1). Again, positive cases of either were negative

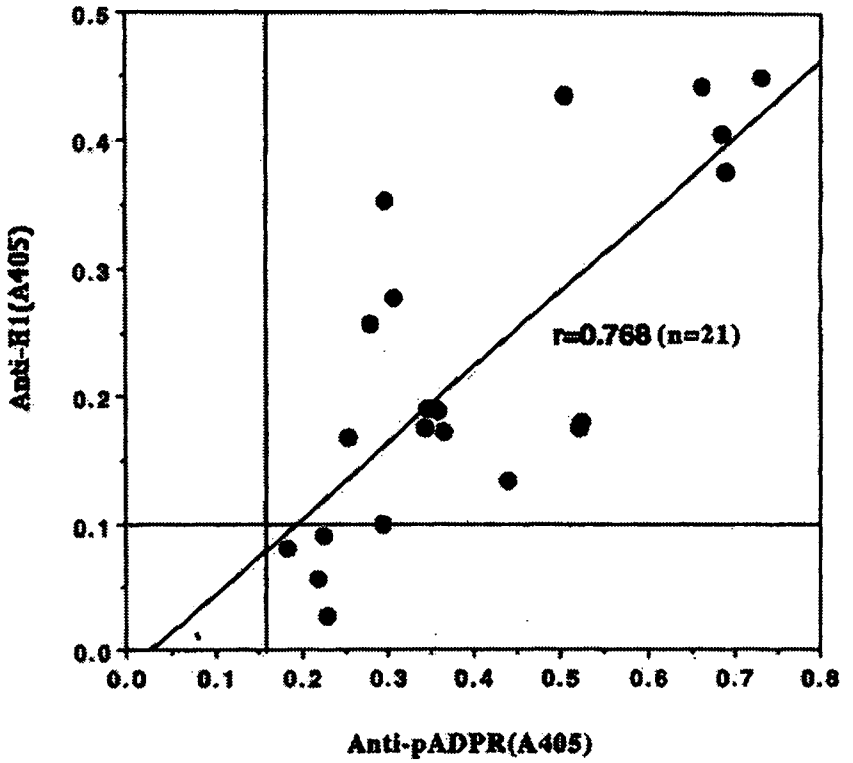


**FIGURE 1.** Correlogram between anti-pADPR antibodies and anti-H1 antibodies in 21 patients with SDAT and AD who showed positive for anti-pADPR antibodies determined by basal level (mean +2 SD) obtained from 28 healthy subjects with age under 60 years as described. Vertical line shows basal level of anti-pADPR (0.173 A405 units) and horizontal line shows that of anti-H1 antibodies (0.105 A405 units). Steep correlation curve in between basal lines is seen ($r = 0.768$).

for anti-dsDNA antibody (data not shown). Whether or not elderly positive for anti-pADPR antibody are prodrome for AD or SDAT is of great concern, therefore we must study carefully the outcome of anti-pADPR antibody-positive elderly.

## DISCUSSION

Given the historical background on pADPR mentioned above, data presented here strongly suggest that H1 is ADP-ribosylated in dementia but not in SLE except for two patients (5%) with high anti-H1 titers (>0.5 A405 units) shown in FIGURE 2. Moreover, the possible presence of ADP-ribosylated H1 on the surface of astrocytes is accessible to the immune system through the

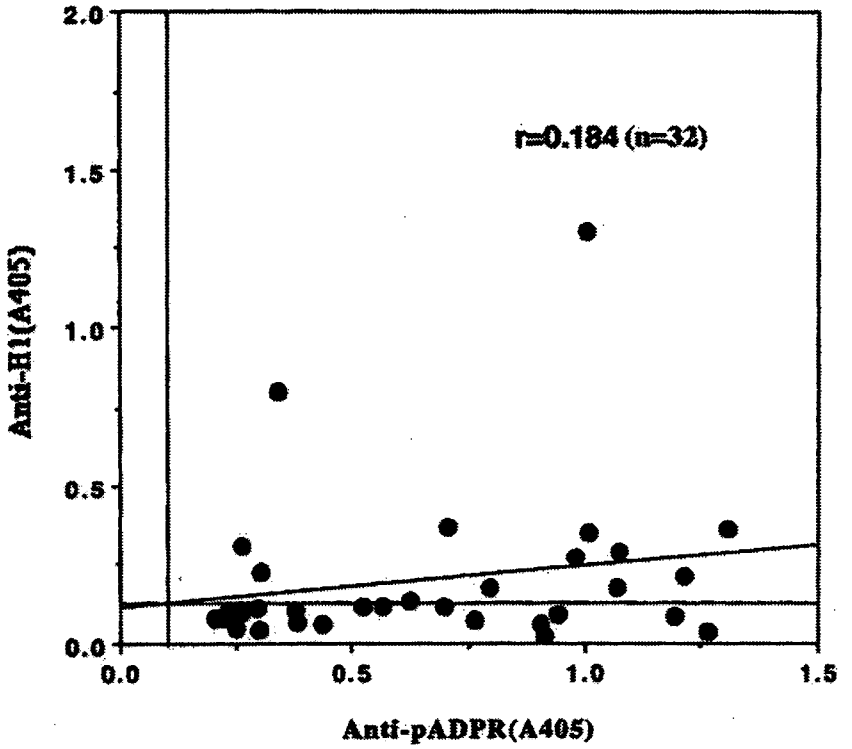


**FIGURE 2.** Correlogram between anti-pADPR antibodies and H1 antibodies in 32 SLE patients positive for anti-pADPR antibodies: basal levels for both antibodies are the same as in FIGURE 1. However, in contrast to cases of SDAT and AD, correlation curve was shallow, indicating low coefficient ($r = 0.187$).

hampered blood–brain barrier (BBB) that is probable in neurodegenerative disorders.[8,9] The difference in antibody subclass between SDAT or AD and SLE, namely the former two cases were IgG1 and IgG2 and the latter was IgG2 alone (data not shown), suggests that the production of autoantibodies occurs under different regulation mechanisms in two diseases. To our understanding, autoantibodies against nuclear antigen-associated autoantigens in degenerative brain diseases have not been specially focused in the field of autoimmunity as well as rheumatic diseases. However, during search for the relationship between neuronal degeneration and autoimmunity in terms of deregulation of BBB, we have found several reports describing the autoimmune responses against not only nuclear chromatins, such as DNA and histones,[10] but also brain-associated antigens.[11,12] It is of note that D'Andrea argues that AD should be listed as an autoimmune disease.[13] Aside from whether or not SDAT and AD fulfill a criterion of autoimmune disease in the critical meaning, it could be excluded that the cases of AD and SDAT presented here are complicated with SLE.

**TABLE 1. Immune responses to chromatins in 59 elderly (77 ± 7.7 years old) without dementia or autoimmune diseases**

| Occurrence of antichromatin antibodies | | | | |
|---|---|---|---|---|
| H1 | nucleosomal DNA | Genomic DNA | Nucleosome | poly(ADP-ribose) |
| 2/59(3) | 4/59(7) | 4/59(7) | 2/59(3) | 22/59(37) |

Parenthesis indicates % positive.

because it seems too high that 80.7% of AD and SDAT patients negative for anti-dsDNA IgG antibody are complicated with SLE or other collagen diseases. Whatever AD or SDAT is an additional autoimmune disease, a parallel assay for IgG autoantibodies against pADPR and H1 would be of use not only as a prodrome of patients with dementia (SDAT/AD) but also for monitoring the disease progress. Moreover, subgrouping of patients with AD and/or SDAT may also be possible according to the presence or absence of both antibodies to pADPR and H1 or single presence of either. In this respect, the findings that 19.3%(5/26) of AD and SDAT patients are negative for anti-pADPR antibodies and that 38.4%(10/26) of these patients are negative for anti-H1 antibodies should be taken into account. Whether or not elderly positive for anti-pADPR antibody mentioned above are a prodrome for AD or SDAT is of great concern, therefore we must carefully examine the outcome of them positive for anti-pADPR antibody. In cases of neuroleptic therapy, such as the phenothiazines to neurodegenerative disease, there happens the occurrence of anti-nuclear antibodies,[14] and, in fact, multicase families with Schizophrenia had anti-dsDNA antibodies,[15] but, we have not used such therapy in patients with AD/SDAT. In conclusion, as Meli *et al.*[16] described, it is quite reasonable that pADPR and/or pADPR polymerase (PARP) plays a key role in excitotoxicity and postischemic brain damage and that pADPR and/or PARP themselves serve as autoantigens as long as BBB is hampered, as proposed.[8,9]

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Review Article

# Strategic targeting of the glucocorticoid receptor for anti-inflammation

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Glucocorticoids are produced in the adrenal cortex under the strict control of the hypothalamus-pituitary-adrenal axis and exert a variety of biological actions including the regulation of glucose and lipid metabolism, electrolyte balance, and modulation of the immune, cardiovascular, and central nervous system. Pharmacologically, glucocorticoids are estimated to be used long-term by 0.5-1% of the general population and up to 2.5% of older adults[1,2]. Despite the established role of glucocorticoids in controlling short-term inflammation, and despite emerging evidence supporting a disease-modifying role in various autoimmune disorders, concern for adverse events associated with glucocorticoids often limits their use. The glucocorticoid compounds bind the glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily, and elicit their pharmacological actions. Recent progress in molecular biology of the GR has extended our understanding of their mechanism of action, however, the molecular basis for the side effects have not been fully clarified. Indeed, dissociation of their therapeutic effects and adverse reactions is still one of the most challenging clinical issues to be solved.

In this lecture, I will focus on the recent understanding of the molecular mechanism of glucocorticoid action and our recent work with ursodeoxycholic acid and cortivazol and discuss rationale to develop novel glucocorticoid-like compounds.

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**Key words** nuclear receptor, transcription, drug, structure

## Current Glucocorticoid Therapy

Since the introduction of glucocorticoids in the treatment of rheumatoid arthritis in 1949[3], scientists and pharmaceutical companies made intensive efforts to maximize the beneficial and to minimize the side effects of the drug. Many synthetic compounds with glucocorticoid activity were produced and the pharmaco-

Table 1 Classification of the nuclear receptor

| | Class I | Class II | Class III |
|---|---|---|---|
| | Steroid hormone receptors | Adopted orphan receptors | Orphan receptors |
| Representative receptors | Receptors for glucocorticoid (GR), estrogen (ER), progesterone (PR), androgen (AR), mineralocorticoid (MR) | Receptors for thyroid hormone (TR), vitamin D (VDR), all-trans retinoic acid (RAR) and the peroxisome proliferator-activated receptor (PPAR) | SF-1, DAX-1, ERRs, Nurs, CoupTFs, SHP, LRH-1, RORs |
| DNA binding form | Homodimer | Heterodimer with 9-cis retinoic acid retinoid X receptor (RXR) | Heterodimer with RXR and/or monomer |
| Response element | Palindrome | Direct repeat | Direct repeat (half site) |

logic differences among these chemicals result from structural alterations of their basic backbone and its side groups. These alterations variably affect the bioavailability of these compounds (i.e., gastrointestinal and/or parenteral absorption, plasma half-life, and metabolism, and interaction with the GR). At first, such modification in the structure succeeded in abolishing mineralocorticoid activity in electrolyte handling. On the other hand, modification of physicochemical characteristics (i.e., water solubility/lipophilicity) is considered for parenteral administration or enhancement of topical potency. Most synthetic glucocorticoids (e.g., prednisolone and dexamethasone) are minimally bound to cortisol-binding globulin and circulate mostly bound to albumin, or in the free form.

The side effects occur only with supraphysiologic doses of glucocorticoids and not with proper replacement, which is equivalent to 12 to 15 mg of hydrocortisone/ $m^2$ body surface area per day[4]. The side effects of glucocorticoids have been shown to be strictly dose-dependent. Thus, as the dosage is escalated to improve efficacy, the side effects also increase. In addition, some side effects are known to be age- and sex-dependent. Major complications are unlikely with short-term treatment (e.g., less than 2 weeks) with high doses of glucocorticoids, although sleep disturbances and gastric irritation are common complaints, and depression, mania, or psychosis, may be infrequently encountered. On the other hand, many side effects are associated with chronic administration of pharmacologic amounts of glucocorticoids. These side effects include the development of varying degrees of the manifestations of Cushing's syndrome[4-6].

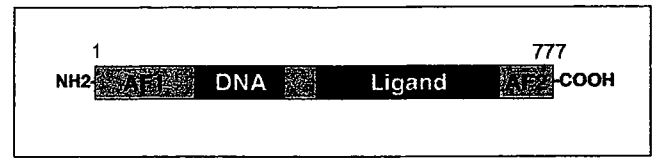


Fig.1 Primary structure of the human glucocorticoid receptor

## GR is A Nuclear Receptor (NR)

NR is a large family of ligand-dependent transcription factors and regulates essential physiological processes including development, reproduction, metabolism, and homeostasis. NRs bind their cognate DNA sequences and modulate gene expression within target tissues. NRs can tentatively be subdivided into three major classes (Table 1). All members of the NR super family share a modular domain structure, consisting of an amino-terminal transcriptional activation function domain (AF1), a conserved zinc-finger DNA binding domain (DBD), a hinge region and a carboxyl-terminal ligand binding domain (LBD) that overlaps with a second transcriptional AF2 domain (Fig.1). Whereas the AF1 activity is constitutive in most cell types or under tissue-specific regulation, the AF2 activity is strictly ligand-dependent[7].

The GR LBD, similar to other NR LBDs, is composed of a-helices and $\beta$-strands folded into a three-layer helical sandwich. The ligand binding pocket is composed of residues from helices 3, 4, 5, 6, 7, 10, and the AF-2 helix as well as residues from $\beta$-strands between helices 5 and 6. Following AF-2 helix is an extended strand that forms a conserved $\beta$-sheet with a $\beta$-strand

between helices 8 and 9. This C-terminal $\beta$-strand also appears to play an important role in receptor activation by stabilizing AF-2 helix in an active conformation. Many AF-2 coactivators for the GR have been identified to date, including steroid receptor coactivator-1 (SRC-1), transcriptional intermediary factor (TIF) 2/GR-interacting protein-1 and cAMP response element binding protein-binding protein (CBP)/p300. These coactivators directly associate with the GR LBD via their LXXLL motif. For example, the LLRYLL sequence in the TIF2 forms a two-turn helix that orients the hydrophobic leucine side chains into a groove formed in part by the AF-2 helix and residues from helices 3, 3', 4, and 5. The N- and C-terminal ends of the coactivator helix are clamped by Glu-755 from the AF-2 helix and Lys-579 in helix 3, respectively. Mutations that disrupt either the first (Glu-755) or the second (Arg-585 and Asp-590) charge clamp dramatically reduce activation mediated by the GR LBD, demonstrating that they are critical for transactivation function of the GR. On the other hand, GR AF-1 coactivators have only recently been described. For example, basal transcription factors including TBP and TFIID have been shown to associate with the AF-1 of GR. TSG101 and DRIP150 have also been reported to interact with GR AF-1 and regulate GR function in a reciprocal manner; GR transcriptional activities are repressed by TSG101 but enhanced by DRIP150. These cofactors are shown to interact with distinct regions of AF-1. Although we now have at hand a large number of regulatory proteins that interact directly or indirectly with the various modular domains of NRs, how ligands differentially regulate the functional interplay between them remains poorly understood[8-10].

Unlike the GR, most nonsteroidal nuclear receptors like PPAR and RAR can interact with corepressors and repress transcription in the absence of ligand or in the presence of antagonists. These corepressors in turn have histone deacetylase activity that trims acetyl groups of nucleosomes, compacting and silencing the promoter to which unliganded nuclear receptor is bound[7].

## GR-Mediated Anti-inflammation

In the absence of ligand, the GR is retained in the cytoplasm in association with chaperone proteins such as heat shock protein 90 (hsp90). Hormone binding initiates the release of the chaperone proteins and translocation of the receptor into the nucleus where GR binds to DNA promoter elements termed glucocorticoid response element (GRE) from which it can usually activate transcription of the target promoter. A dozens of the target genes of the GR have been identified, most of which transmit metabolic effects of glucocorticoids. A few genes are shown to be

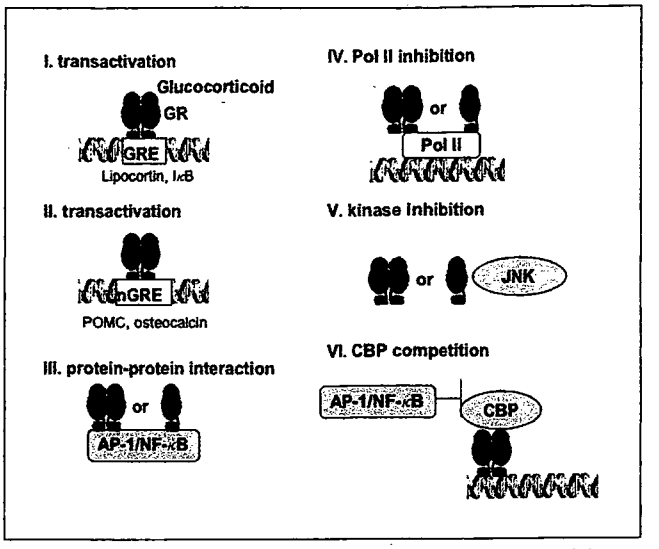


Fig.2 Multiple mechanisms for glucocorticoid receptor (GR)-mediated antiinflammation.

related anti-inflammatory properties of glucocorticoids. On the other hand, transcriptional repression activity is central to the glucocorticoid-mediated anti-inflammation and antiproliferative effects, and a number of transcriptional repression mechanisms mediated by GR have been described (Fig.2). Of note, in contrast to other classes of NR, repression by steroid receptors occurs only in the presence of ligand. The mechanism of repression varies significantly and ranges from effects at the level of DNA to effects on RNA polymerase and transcriptional elongation, and transcription factors directly. These include mechanisms dependent on either GR-DNA or GR-protein interactions. The variety of potential interactions suggests exquisite control over repression that is highly context-dependent. Initial studies emphasized that repression of proinflammatory transcription factors including AP-1 and NF-kB may be central in GR-mediated anti-inflammation[8,9].

## Development of Novel Glucocorticiod-like Compounds

Based on the development of molecular biology of the NRs, steroid pharmacology is increasingly focused in the development of novel ligands with selective modulatory activities[11]. Since dissociation of the side effects of glucocorticoids would definitely contribute to medical fields, GR could be one of the rational molecular targets for such purpose. It is obvious that a novel agent must have the same efficacy in such diseases for which glucocorticoids are currently-indicated, but with reduced side effects. Identification of novel GR ligands have resulted in a

number of divergent terminologies, and Rosen and Miner recently provided putative definitions of some of the key terms[12]:

- SGRM (selective GR modulator) and SeGRA (selective GR agonist). Both SGRM and SeGRA are general class descriptors used to describe compounds with an improved therapeutic index *in vivo* by whatever mechanism.
- Gene-selective compound. This term refers to compounds that act on the receptor to alter gene expression in a gene- or promoter-specific fashion. In other words, some genes might be activated, some might be repressed, but the resulting profile differs from that of currently used glucocorticoids.
- Dissociated compound. This term is usually used to refer to a compound that "dissociates" activation from repression. Compounds in this class fail to globally activate gene expression, but still significantly repress transcription.
- Soft steroids/glucocorticoids (also known as "antedrugs"). This describes corticosteroids that act at or near the site of administration but are inactivated by enzymes, thereby reducing systemic exposure and activity. These are often described for topical and inhaled therapies that act locally but are rapidly metabolized once they enter systemic circulation.

G. Schütz and his colleagues, using dimerization-deficient mutant GR that prevent gene activation by GR but do not affect repression, showed that the anti-inflammatory activity of steroids was maintained and suggested that repression may be sufficient for anti-inflammatory activity[13]. Although a variety of compounds have been tested in terms of dissociation of activation and repression, at this moment a complete dissociated compound is not available. Moreover, such compounds which were once shown to be a dissociated one *in vitro*, often failed to decrease glucocorticoid's metabolic side effects with keeping its anti-inflammatory activities *in vivo*. The other compounds fail to keep their dissociated characteristics at higher, or therapeutic concentrations. Given the battery of genes and the multitude of potential regulatory mechanisms, finding a compound that actually separates all activated genes from all repressed genes seems highly unlikely. It is also unclear whether such a compound would be truly desirable because activation of anti-inflammatory genes may also play a role in the treatment of inflammatory diseases. Of the proposed dissociated compounds that have been published, all have been shown to differentially regulate one or sometimes two genes. This is not the same as demonstrating that the compound is dissociated on all glucocorticoid target genes. It, however, should be noted that the activation/repression hypothesis has provided a very useful framework to find novel compounds with potential utility, and some success has already been achieved at least preclinically[12].

Recent structural analyses of the nuclear receptors establish a paradigm of receptor activation, in which agonist binding induces the LBD/AF-2 helix to form a charge clamp for coactivator recruitment[10]. However, these analyses have not sufficiently addressed the mechanisms for differential actions of various synthetic steroids in terms of fine tuning of multiple functions of whole receptor molecules. In this line, our studies with two distinct compounds, ursodeoxycholic acid (UDCA) and cortivazol (CVZ), provided the rationale for ligand-based selective modulation of the receptor activities.

## Ursodeoxycholic Acid (UDCA)

UDCA is the current mainstay of treatment for various liver diseases including primary biliary cirrhosis, autoimmune hepatitis, and hepatitis C. UDCA has multiple functions, acting not only as a bile secretagogue, but also as a cytoprotective agent, immunomodulator, and inhibitor of cellular apoptosis[14-18]. Based on this cumulative evidence of the cytoprotective and immunomodulatory effects of UDCA, we tried to identify the target molecule and pathway of UDCA action. It was shown that UDCA specifically translocates the GR into the nucleus as a DNA binding species but does not elicit its transactivational function in a transient transfection assay (Fig.3). Moreover, the LBD of the GR is responsible for UDCA-dependent nuclear translocation of the GR. Indeed, we demonstrated that UDCA acts on the distinct region of the LBD when compared with the classical GR agonist dexamethasone, resulting in loss of coactivator recruitment and differential regulation of gene expression by the GR (Fig.4). Our data clearly indicated that UDCA, at least in part via activation of the GR, suppresses NF-$\kappa$B-dependent transcription through the intervention of GR-p65 interaction[19,20]. Together with the established clinical safety of UDCA, we may propose that UDCA could be a prototypical compound for development of a novel and selective GR modifier. Recently, using a flourescently labeled UDCA molecule, we showed that UDCA shows similar distibution pattern with GR in hepatocytes and that GR is crucial for the nuclear translocation of UDCA for reducing apoptosis[21]. In fact, it is now wellknown that bile acids are ligands of a various NR including the farnesoid X-activated receptor (FXR) and VDR[22-25]. Our understanding the physiological role of bile acids, thus, is still expanding and NR-target drug development may provide a novel milieu for treatment of various gastrointestinal diseases.

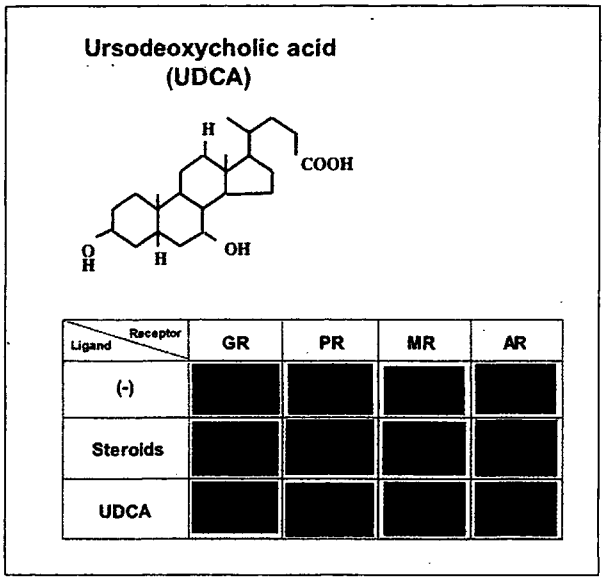


Fig.3 GR-selective activation by ursodeoxycholic acid (UDCA)

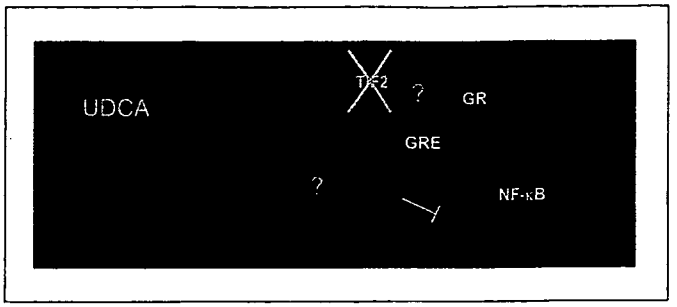


Fig.4 Proposed mechanism of UDCA in GR-mediated immunomodulation

## Cortivazol (CVZ)

The phenylpyrazologlucocorticoid CVZ is a unique synthetic glucocorticoid agonist with complex binding properties and is more potent than DEX[6] (Fig.5). We demonstrated that CVZ selectively binds to the GR but not to the MR (Fig. 6) and, based on two criteria, we proposed that the functional interaction of CVZ with the GR LBD is different from that of DEX (Fig.7). Firstly, deletion of the last 12 amino acids of GR severely compromises DEX but not CVZ binding and secondly, the point mutant L753F, in which Leu-753 in AF-2 is substituted to Phe, can efficiently recruit TIF2 to the LBD when bound to CVZ but not when bound to DEX[27]. These results prompted us to propose that occupancy of the GR LBD by CVZ might lead to a more stable active conformation that can tolerate the disrupting effects of LBD mutations and may have unique effects on the structure and function of the whole GR molecule. Structural docking analysis revealed that although CVZ is more bulky than other agonists, it can be accommodated in the ligand binding pocket of the GR by reorientation of several amino acid side chains but without major alterations in the active conformation of the LBD. In this induced fit model, the phenylpyrazole A-ring of CVZ establishes additional contacts with helices 3 and 5 of the LBD that may contribute to a more stable LBD configuration. Structural and functional analysis revealed that CVZ is able to compensate for the deleterious effects of a C-terminal deletion of the LBD in a manner that mimics the stabilizing influence of the F602S point mutation. CVZ-mediated productive recruitment of TIF2 to the C-terminally deleted LBD requires the receptor's own DNA binding domain and is positively influenced by the N-terminal regions of GR or progesterone receptor. These results support a model where ligand-dependent conformational changes in the LBD play a role in GR-mediated gene regulation via modular interaction with the DBD and AF-1. Steroid pharmacology is increasingly focused in the development of ligands with selective modulatory activities. Because the mode of interdomain communication may be distinct for each receptor and may be modulated in a ligand-, tissue-, and promoter-context-dependent manner, ligands such as CVZ and other phenylpyrazole analogs that manipulate this regulatory avenue will not only provide a better understanding of the mechanisms of interdomain communication but also provide novel leads in the development of selective GR modulators[28]. Indeed, a further series of compounds based on an arylpyrazole structure have recently been published. These compounds specifically bind the GR with relatively high affinity. These compounds differ in their relative activity to inhibit cell-based assays of proliferation, adipocyte differentiation or osteoblast differentiation. Moreover, transcriptome profile analyses revealed that the different molecular structures have differential effects on individual target genes. It was striking that, as also indicated in the case of CVZ, subtle changes in structure of the ligand caused markedly distinct GR regulatory effects in more than one cell line. Chromatin immunoprecipitation assays suggested that the different compounds alter the relative affinity of the GR for specific DNA sequences. The authors concluded that the induced structure of the LBD of the GR appears to influence the interaction with DNA sequence and thereby specify a distinct profile of gene regulatory events[29]. This study overall supports the idea that pursuit of the perfect dissociating glucocorticoid ligand may well be complicated, but it is certainly possible that even an imperfectly dissociating compound may be more than sufficient to offer an improved therapeutic index and thereby

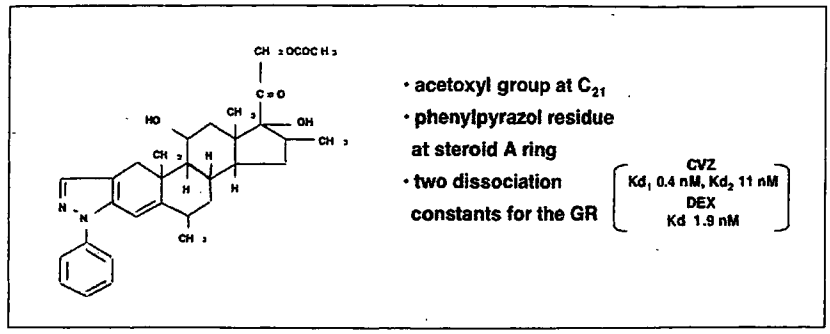


Fig.5 Characteristics of cortivazol (CVZ)

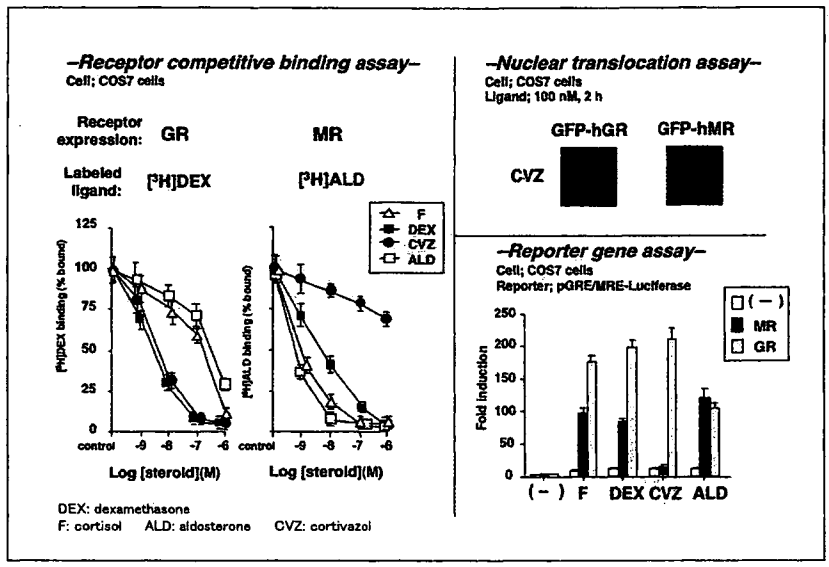


Fig.6 CVZ is a specific ligand for the GR

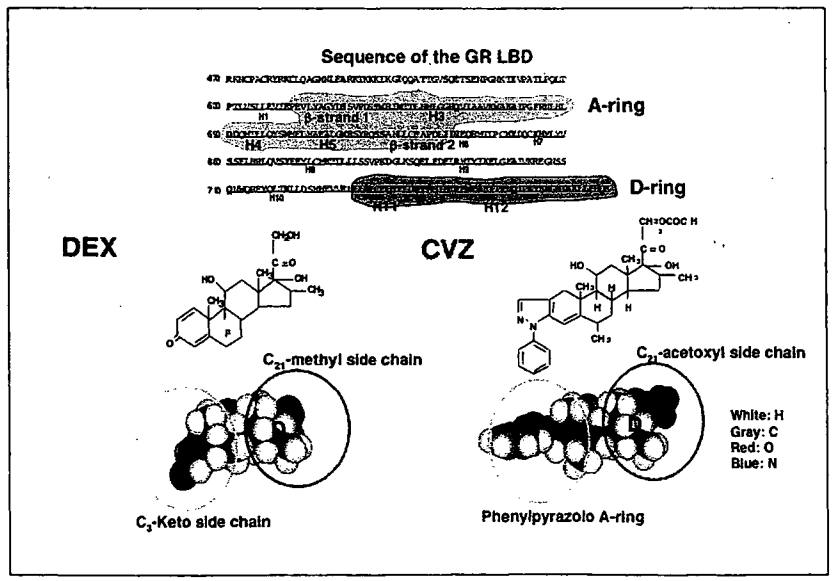


Fig.7 Distinct binding poperties of CVZ to the GR -- comparison with dexamethasone (DEX)

unleash the full anti-inflammatory potency of glucocorticoids without the side-effect profile.

## Future Directions

A safer glucocorticoid should have full efficacy in anti-inflammatory activity, but reduced efficacy and potency in one or more side effects. The development of new safer anti-inflammatory

agents that target the GR is now gaining momentum after years of work on steroids and, more recently, nonsteroidal molecules. The molecular details behind the action of the newer compounds being described may point the way to more effective assays capable of detecting novel anti-inflammatory agents.

The detection of a tissue selective or a functionally selective ligand for the GR will be difficult, and there is no guarantee, once such a ligand is found, that it will have the necessary profile *in vivo*. However, recent reports of SGRMs with equal efficacy and improved side effect profiles compared with steroids together with molecular discoveries of the receptor mechanism of action provide fertile ground for additional efforts. Thus, despite the difficulties associated with developing a novel glucocorticoid, progress in this area would be a major benefit to the large number of patients suffering from the side effects of steroids, but needing the anti-inflammatory and anti-cancer activity to maintain their quality of life.

Acknowledgement

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# Humanized Anti-CD26 Monoclonal Antibody as a Treatment for Malignant Mesothelioma Tumors

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**Abstract**

**Purpose:** CD26 is a 110-kDa cell surface antigen with a role in tumor development. In this report, we show that CD26 is highly expressed on the cell surface of malignant mesothelioma and that a newly developed humanized anti-CD26 monoclonal antibody (mAb) has an inhibitory effect on malignant mesothelioma cells in both *in vitro* and *in vivo* experiments.

**Experimental Design:** Using immunohistochemistry, 12 patients' surgical specimens consisting of seven malignant mesothelioma, three reactive mesothelial cells, and two adenomatoid tumors were evaluated for expression of CD26. The effects of CD26 on malignant mesothelioma cells were assessed in the presence of transfection of CD26-expressing plasmid, humanized anti-CD26 mAb, or small interfering RNA against CD26. The *in vivo* growth inhibitory effect of humanized anti-CD26 mAb was assessed in human malignant mesothelioma cell mouse xenograft models.

**Results:** In surgical specimens, CD26 is highly expressed in malignant mesothelioma but not in benign mesothelial tissues. Depletion of CD26 by small interfering RNA results in the loss of adhesive property, suggesting that CD26 is a binding protein to the extracellular matrix. Moreover, our *in vitro* data indicate that humanized anti-CD26 mAb induces cell lysis of malignant mesothelioma cells via antibody-dependent cell-mediated cytotoxicity in addition to its direct anti-tumor effect via $p27^{kip1}$ accumulation. *In vivo* experiments with mouse xenograft models involving human malignant mesothelioma cells show that humanized anti-CD26 mAb treatment drastically inhibits tumor growth in tumor-bearing mice, resulting in enhanced survival.

**Conclusions:** Our data strongly suggest that humanized anti-CD26 mAb treatment may have potential clinical use as a novel cancer therapeutic agent in CD26-positive malignant mesothelioma.

Malignant mesothelioma is an aggressive cancer arising from the mesothelial cells lining the pleura. It is usually associated with the history of chronic asbestos exposure (1). Because of the long latency period between asbestos exposure and tumor development, the annual incidence of 2,500 new cases in the United States is expected to increase by >50% in the coming decade (2). Moreover, incidence world wide is projected to increase substantially in the next decades (3). The prognosis is very poor with a median survival of 4 to 12 months despite the therapies currently used, inducing surgery, radiotherapy, and chemotherapy (4). Because of the inefficacy of the conventional treatments, novel therapeutic strategies are urgently needed to be developed.

CD26 is a 110-kDa surface glycoprotein with dipeptidyl peptidase IV activity able to cleave selected biological factors to alter their functions (5). CD26/dipeptidyl peptidase IV is involved in T-lymphocyte costimulation and signal transduction processes (6, 7) and regulates topoisomerase II α level in hematologic malignancies, affecting sensitivity to doxorubicin and etoposide (8). Expressed on various tissues (4, 9), CD26 is involved in the development of certain human cancers (9–12). CD26 is also known to serve as a binding motif for extracellular matrix (ECM) in human and rodents (13, 14). Previously, we reported that CD26 was collagen-binding protein using a CD26 positive JMN cell line, which is derived from malignant mesothelioma (15). Moreover, our previous works have shown that anti-CD26 monoclonal antibody (mAb) inhibits growth of CD26-positive T-cell malignancies (16, 17) and renal cell carcinoma (18).

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**Conflict of interest:** Dr. Morimoto is a board member of Y's Therapeutics, and Dr. Dang is a scientific adviser in Y's Therapeutics. The other authors have no competing financial interests.

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Our previous report shows that the murine anti-CD26 mAb 14D10, which recognizes the cell membrane-proximal glycosylated region starting with a 20-amino acid flexible stalk region of human CD26, has direct antitumor effect by inducing $G_1$-S arrest while concomitantly blocking the adhesion of cancer cells to the ECM. However, another murine anti-CD26 mAb, termed 5F8, which detects the cysteine-rich domain of CD26, lacks this biological activity (18).

Because human malignant mesothelioma is a highly malignant tumor resistant to apparent conventional treatment, the detection of novel target and development of new treatment strategies in malignant mesothelioma are urgently needed (4, 19). In this report, we analyzed the expression of CD26 in the tissues of patients with malignant mesothelioma and validated the antitumor effect of a novel humanized anti-CD26 mAb which was constructed from high-affinity Fab clone to the 14D10 variable region by targeting malignant mesothelioma, hence concomitantly showing the functional role of CD26 in this neoplasm.

## Materials and Methods

***Reagents and antibodies.*** Anti-CD26 mouse mAb (IgG1)14D10, 5F8, and anti-CD45RA mouse mAb (IgG1) 2H4 were developed in our laboratory as described previously (20, 21), with the last one being used as control. Normal human IgG1 (Sigma-Aldrich) was also used as a control. Humanized anti-CD26 mAb (IgG1 isotype) was constructed from 14D10 coding sequence (generously provided by Y's Therapeutics). Mouse mAb to PKBα/Akt, CDK2, CDK4, CDK6, cyclin E, and β-actin were from Cell Signaling Technology Inc., and mouse mAb to $p27^{kip1}$, $p21^{cip1/waf1}$, cyclin D1, and activated caspase-3 were from BD PharMingen. Antihuman IgG, Fcγ fragment specific F(ab')2 fragment of goat and anti-mouse IgG, Fcγ fragment specific F(ab')2 fragment of goat were from Jackson ImmunoResearch.

***Cell culture and transfection.*** JMN cells were a kind gift from Dr. Brenda Gerwin (Laboratory of Human Carcinogenesis, NIH, Bethesda, MD). NCI-H2452 and 293T cells were obtained from the American Type Culture Collection. JMN and NCI-H2452 cell lines were derived from patients with malignant mesothelioma. All cells were grown in RPMI medium (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL; Life Technologies) or G418 (500 μg/mL; Sigma-Aldrich). 293T cells were transfected with full-length CD26 subcloned into a pEB6 vector (22) using FuGENE6 reagent (Roche Diagnostics).

***2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium assay.*** Cells were subjected to incubation in 96-well plates in media alone or in the presence of humanized anti-CD26 mAb (0.1, 1.0, or 10 μg/mL) or 2H4 (0.1, 1.0, or 10 μg/mL) for a total volume of 100 μL ($5 \times 10^3$ cells per well). After 24 h of incubation in 37°C, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Seikagaku) was added to each well. After another 2 h of incubation, water soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-methoxy-5-methylphenazinium, was measured at 450 nm using a microplate reader (Bio-Rad). All samples were tested in triplicate. Values reported represent the means of triplicated wells, and SE was within 15.

***Immunohistochemistry.*** For immunohistochemistry, 12 patients' surgical specimens consisting of seven malignant mesothelioma, three reactive mesothelial cells, and two adenomatoid tumors were evaluated. For each, 10% formalin-fixed, paraffin-embedded specimens, containing both the carcinoma and its adjacent nonneoplastic tissue, were prepared. Paraffin-embedded tissues were dewaxed and rehydrated using xylene and ethanol, respectively. Slides were deparaffinized, then heated in a microwave processor for antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After blocking in 3% (v/v) bovine serum albumin, slides were incubated at 4°C overnight with the primary antibody (anti-CD26 mAb) and washed with PBS and the secondary antibody was labeled with biotin and applied for 30 min. Streptavidin-LSA amplification method was carried out for 30 min followed by peroxidase/diaminobenzidine substrate/chromagen. The slides were counterstained with hematoxylin. Two different pathologists checked the validity of the obtained results. All human specimens were obtained from Department of Pathology, Keio University (Tokyo, Japan), and informed consents were obtained from all patients according to the format of the institutional review board.

***Depletion of endogenous CD26.*** To deplete endogenous CD26, small interfering RNA (siRNA) oligo-targeting CD26 cDNA (accession no. NM_001935) was made according to the design site of TAKARA BIO;[5] sense: 5'-GAAAGGUGUCAGUACUAUU TT-3', antisense: 3'-TT CUUUCCACAGUCAUGAUAA-5', with scrambled control of small interfering RNA oligo-targeting human Cas-L; sense: 5'-UAAUUAGG-GUCGGGUAAAC TT-3', antisense: 3'-TT AUUAAUCCCAGCCCA-UUUG-5' being used as control. CD26 siRNA oligo (siCD26) was transfected using TransIT-TKO transfection reagent (Mirus Bio Corporation) according to the manufacturer's protocol.

***SDS-PAGE and immuno-blotting.*** Preparation of whole-cell lysates, cell fractionations, and SDS-PAGE were done as described elsewhere (23).

***Antibody-dependent cell-mediated cytotoxicity.*** The capacity of mAb to induce effector cell-dependent lysis of tumor cells was evaluated in Calcein-AM-release assay. Healthy donor natural killer cells were isolated from peripheral blood mononuclear cells by NK Cell Isolation kit II Miltenyi Biotec (Bergisch Gladbach) and used as effector cells. Target cells ($1 \times 10^6$) were labeled with 10 μmol/L Calcein-AM (Dojindo) under shaking conditions at 37°C for 1 h. Cells were washed thrice with PBS and were resuspended in culture medium ($1 \times 10^5$ cells/mL). Labeled cells were dispensed in 96-well U-bottomed plates ($5 \times 10^3$ in 50 μL/well) and preincubated (37°C, 30 min) with 50 μL of 7-fold serial dilutions of humanized anti-CD26 mAb or 14D10 in culture medium, ranging from 0.1 pg/mL to 0.1 mg/mL (final concentrations). Culture medium was added instead of mAb to determine the spontaneous Calcein-AM release, with Triton X-100 (1% final concentration) being added to determine the maximal Calcein-AM release. Thereafter, human effector cells (HuEC) were added to the wells ($5 \times 10^5$ cells per well) and cells were incubated at 37°C overnight. Supernatants were then collected for measurement of the Calcein-AM release. Percentage of specific lysis was calculated using the following formula: % specific lysis = (experimental release - spontaneous release)/(maximal release - spontaneous release) × 100; where maximal release was determined by adding Triton X-100 to target cells and spontaneous release was measured in the absence of sensitizing Abs and effector cells.

***Complement-dependent cytotoxicity.*** Complement-dependent cytotoxicity (CDC) assay was done as described previously (24). Target cells were dispensed in 96-well U-bottomed plates ($1 \times 10^5$ cells per well) incubated with various concentrations of mAbs at 4°C for 30 min. Subsequently, human serum was added and cells were incubated at 37°C for 2 h. Evaluation of CDC-specific cell death along with antibody-dependent cell-mediated cytotoxicity (ADCC)-specific cell death was assessed by Annexin V-FITC Apoptosis Detection kit (BioVision) and detection of activated caspase-3.

***Assessment of antitumor activity of humanized anti-CD26 mAb in effector-depleted SCID mice.*** All *in vivo* studies were approved by the Institute Animal Care and Use Committee. Six-week-old female NOD-SCID mice were purchased from Charles River (Kanagawa, Japan) and were pretreated with anti-asialo-GM1 polyclonal antisera 25% (v/v; WAKO) i.p. 1 day before mAb treatment.

[5] http://www.takara-bio.co.jp/RNAi.htm

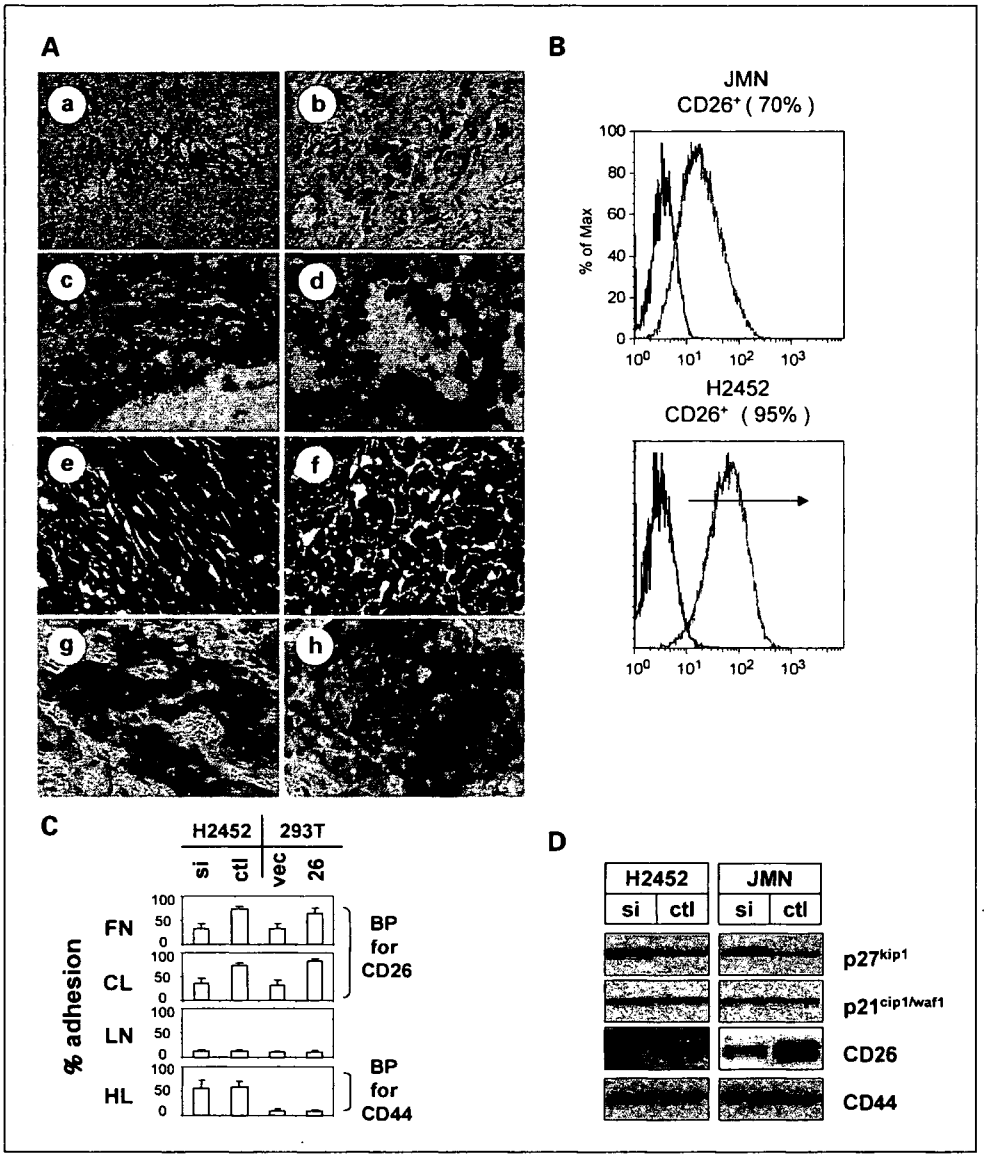


Fig. 1. Expression and functional role of CD26 in malignant mesothelioma. *A*, immunochemical localization of CD26 in adenomatoid tumor, reactive mesothelial cells and malignant mesothelioma. *a*, CD26 in adenomatoid tumor; *b*, CD26 in reactive mesothelial cells; *c*, CD26 in localized malignant mesothelioma; *d*, CD26 in well-differentiated papillary malignant mesothelioma; *e* and *f*, H&E stain in diffuse malignant mesothelioma; *g and h*, CD26 in diffuse malignant mesothelioma. Diffuse malignant mesothelioma specimens are showing biphasic features of sarcomatous malignant mesothelioma (*f, h*) and epithelial malignant mesothelioma (*g, i*). Indicated panels are representative of 12 consecutive specimens. Original magnification, ×100. *B*, surface expression of CD26 on mesothelioma cell lines was analyzed by flow cytometry. Gray line, CD26 histograms were obtained by staining mouse anti-CD26mAb (14D10) followed by staining with rabbit anti-mouse Igb FITC conjugate; black line, control histograms represent back ground fluorescence obtained by staining of isotype-matched control mAb (2H4). *C*, adhesive property of CD26 to ECM. CD26-depleted NCI-H2452 (*si*), scrambled control oligo-transfected NCI-H2452 (*ctl*), pEB6 vector – transfected 293T (*vec*), or pEB6-CD26 – transfected 293T (26) were plated onto 60-mm dishes ($2 \times 10^6$ cells per dish) coated with fibronectin (*FN*), collagen I (*CL*), laminin (*LN*), or hyaluronan (*HL*) and cultured for 18 h. Fibronectin and collagen I are binding proteins (*BP*) to extracellular region of CD26, with hyaluronan being binding protein for CD44. The adhesive ability of cancer cells was expressed as the mean number of cells that had attached to the bottom surface of the dish. Columns, number of cells per field of view; bars, SD. Values for adhesion were determined by calculating the average number of adhesive cells per squared millimeters over three fields per assay and expressed as an average of triplicate determinations. Adhesive cells (%): adhesive cells/adhesive cells + nonadhesive cells. *D*, depletion of CD26 elicits up-regulation of $p27^{kip1}$. NCI-H2452 cells and JMN cells were transfected with siRNA oligo (*si*) of CD26 or control oligo (*ctl*). At 48 h after transfection, cells were harvested, lysed, and subjected to SDS-PAGE, then probed by specific antibody to $p27^{kip1}$, $p21^{cip1/waf1}$, CD26, and CD44.

To assess the effect of humanized anti-CD26 mAb against tumorigenicity, JMN cells ($1 \times 10^6$) were inoculated s.c. into the left flank of mice. Mice were treated with intratumoral injection of isotype-matched control mAb and 5F8, 14D10, or humanized anti-CD26 mAb (10 μg per each injection) on the 14th day after cancer cell inoculation when the tumor mass became visible (5 mm in size). Each mAb was given thrice per week. Tumor-bearing mice were then monitored for tumor development and progression. Tumor size was determined by caliper measurement of the largest ($x$) and smallest ($y$) perpendicular diameters and was calculated according to the formula $V = \pi/6 \times xy^2$.

To assess the effect of humanized anti-CD26 mAb against tumor dissemination, JMN cells ($1 \times 10^5$) were injected i.v. via tail vein. Thereafter, mice were treated with i.v. injection of isotype-matched control mAb and 5F8, 14D10, or humanized anti-CD26 mAb (10 μg per each injection), starting on the day of cancer cell injection. Each mAb was given thrice per week. Cumulative proportion survival was assessed by Kaplan-Meier.

**Table 1.** CD26 expression profile in patient samples

| Patient no. | Gender/Age | Origin | Histology | CD26 | |
|---|---|---|---|---|---|
| | | | | CS | C |
| 1 | M/55 | Pleura | RMC | - | ± |
| 2 | F/63 | Pleura | RMC | - | ± |
| 3 | M/58 | Pleura | RMC | - | ± |
| 4 | F/39 | Ovary | AT | - | + |
| 5 | F/5 | Ovary | AT | - | ± |
| 6 | M/67 | Pleura | MM | + | ++ |
| 7 | M/60 | Pleura | MM | ++ | +++ |
| 8 | M/49 | Pleura | MM | + | ++ |
| 9 | F/74 | Pleura | MM | - | ++ |
| 10 | M/50 | Pleura | MM | ++ | ++ |
| 11 | M/77 | Pleura | MM | + | +++ |
| 12 | M/61 | Pleura | MM | + | +++ |

Abbreviations: RMC, reactive mesothelial cell; AT, adenomatoid tumor; MM, malignant mesothelioma; CS, cell surface; C, cytoplasm.

*Assessment of antitumor activity of humanized anti-CD26 mAb in effector-present Balb mice.* Six-week-old female Balb mice were purchased from Charles River, and treatment with anti-asialo-GM1 polyclonal antisera was not introduced to preserve the binding of the mouse effector system.

To assess the effect of humanized anti-CD26 mAb against tumorigenicity, JMN cells ($1 \times 10^6$) were inoculated s.c. into the left flank of mice. Mice were treated with intratumoral injection of isotype-matched control mAb and 5F8, 14D10, or humanized anti-CD26 mAb (10 μg per each injection) on the 14th day after cancer cell inoculation when the tumor mass became visible (5 mm in size). Each mAb was given thrice per week. Tumor-bearing mice were then monitored for tumor development and progression. Tumor size was determined by caliper measurement of the largest ($x$) and smallest ($y$) perpendicular diameters and was calculated according to the formula $V = \pi/6 \times xy^2$. On the 35th day after the first mAb treatment, all mice were euthanized to assess the microscopic feature of resected specimens in s.c. tumorigenicity model.

To assess the effect of humanized anti-CD26 mAb against tumor dissemination, JMN cells ($1 \times 10^5$) were i.v. injected via tail vein. Thereafter, mice were treated with i.v. injection of isotype-matched control mAb or humanized anti-CD26 mAb (10 μg per each injection) starting on the day of cancer cell injection. Each mAb was given thrice per week. Cumulative proportion of survival was assessed by Kaplan-Meier. To further assess the effect of humanized anti-CD26 mAb on distant metastasis formation, treated mice were euthanized and multiple metastasis formation in the lung and liver was calculated in another tumor dissemination model. JMN cells ($1 \times 10^5$) were injected i.v. into mice in each group. Mice were treated with i.v. injection of isotype-matched control mAb (lane 1, $n = 4$), 5F8 (lane 2, $n = 4$), 14D10 (lane 3, $n = 4$), or humanized anti-CD26 mAb (lane 4, $n = 4$) on the day of cancer cell injection. Each mAb was given thrice per week. On the 35th day after cancer cell injection, mice were euthanized and multiple metastasis formation in the lung and liver was calculated.

*Construction of HuEC-engrafted mice and assessment of antitumor activity in NOD/Shi-scid. IL-Rγ$^{null}$ mice.* NOD/Shi-scid. IL-Rγ$^{null}$ (NOG mice) were obtained from Central Institute for Experimental Animals. Human peripheral blood mononuclear cells were isolated from the peripheral blood of a healthy donor using Lymphoprep (AXIS-SHIELD) and were used as HuEC. Thereafter, HuEC ($5 \times 10^6$ cells) were injected i.p. in a volume of 0.2 mL suspended in PBS into NOG-SCID mice under sterile conditions. The mice were pretreated with a 0.2 mL anti-asialo-GM1 polyclonal antisera 25% (v/v; WAKO) given i.p. 1 day before HuEC injection. NCI-H2452 cells ($5 \times 10^4$) were injected i.p. into SCID mice engrafted with human HuEC 1 day after HuEC injection. One, three, and five days later, humanized anti-CD26 mAb were injected i.p. Mice were observed daily to monitor for death due to ascites tumor development. Cumulative proportion of survival was assessed by Kaplan-Meier.

## Results

*Cell surface CD26 is highly expressed on human malignant mesothelioma.* We first evaluated CD26 expression level on surgically resected human malignant mesothelioma tissues from patients. Twelve consecutive surgically resected specimens from the primary sites were examined for cell surface CD26 expression. CD26 was highly expressed on all malignant mesothelioma tissues (Fig. 1A; Table 1). In adenomatoid tumor or reactive mesothelial cells, CD26 expression was very weak (Fig. 1A-a,b). In contrast, CD26 was highly expressed in various pathologic types of malignant mesothelioma, including localized malignant mesothelioma, well-differentiated papillary malignant mesothelioma, and diffuse malignant mesothelioma (Fig. 1A-c to h). These results suggested that CD26 is highly expressed in malignant mesothelioma but not in benign mesothelial tissues.

*CD26 plays a role in cell adhesion to ECM.* Malignant mesothelioma cell lines, JMN and NCI-H2452, exhibited high-surface CD26 expressions (Fig. 1B).

Because CD26 has been described previously to play a role in cell adhesion to the ECM proteins (13, 25), we examined whether CD26 plays a role in cellular interaction with the ECM. As seen in Fig. 1C, NCI-H2452 that were depleted of endogenous CD26 using siRNA oligo showed significant loss of CD26 binding to ECM proteins, including fibronectin and collagen I. In contrast to these results, depletion of CD26 did not alter binding to laminin (an ECM protein lacking binding ability to CD26) or hyaluronan (a ligand for CD44; Fig. 1C). In further support of these findings, 293T cells transfected with full-length CD26 cDNA subcloned into pEB6 vector showed higher binding ability to fibronectin and collagen I than control pEB6-transfected 293T cells (Fig. 1C). Moreover, depletion of CD26 was associated with the up-regulation of $p27^{kip1}$ (Fig. 1D). These findings thus suggested that CD26 serves as a binding molecule to distinct ECM proteins and that contact inhibition may play a contributing role to the observed CD26 depletion-mediated up-regulation of $p27^{kip1}$ associated with CD26 depletion (26, 27).

*Anti-CD26 mAb perturbs cellular binding to ECM.* Because CD26 proved to be an ECM-binding protein, we further evaluated whether anti-CD26 mAbs disrupt cellular adhesion to ECM. For this purpose, isotype-matched control mAb and 5F8, 14D10, and humanized anti-CD26 mAb were evaluated for potential disruption to cellular adhesion to ECM. As seen in Fig. 2A, JMN cells treated with 14D10 and humanized anti-CD26 mAb had decreased binding to fibronectin and collagen I, whereas control mAb and 5F8 (anti-CD26 mAb without biological function) did not influence binding to fibronectin and collagen I. Moreover, 14D10 and humanized anti-CD26 mAb transmitted direct growth inhibition to JMN cells by *in vitro* proliferation assay in a dose-dependent manner, with humanized anti-CD26 mAb having a stronger antiproliferative

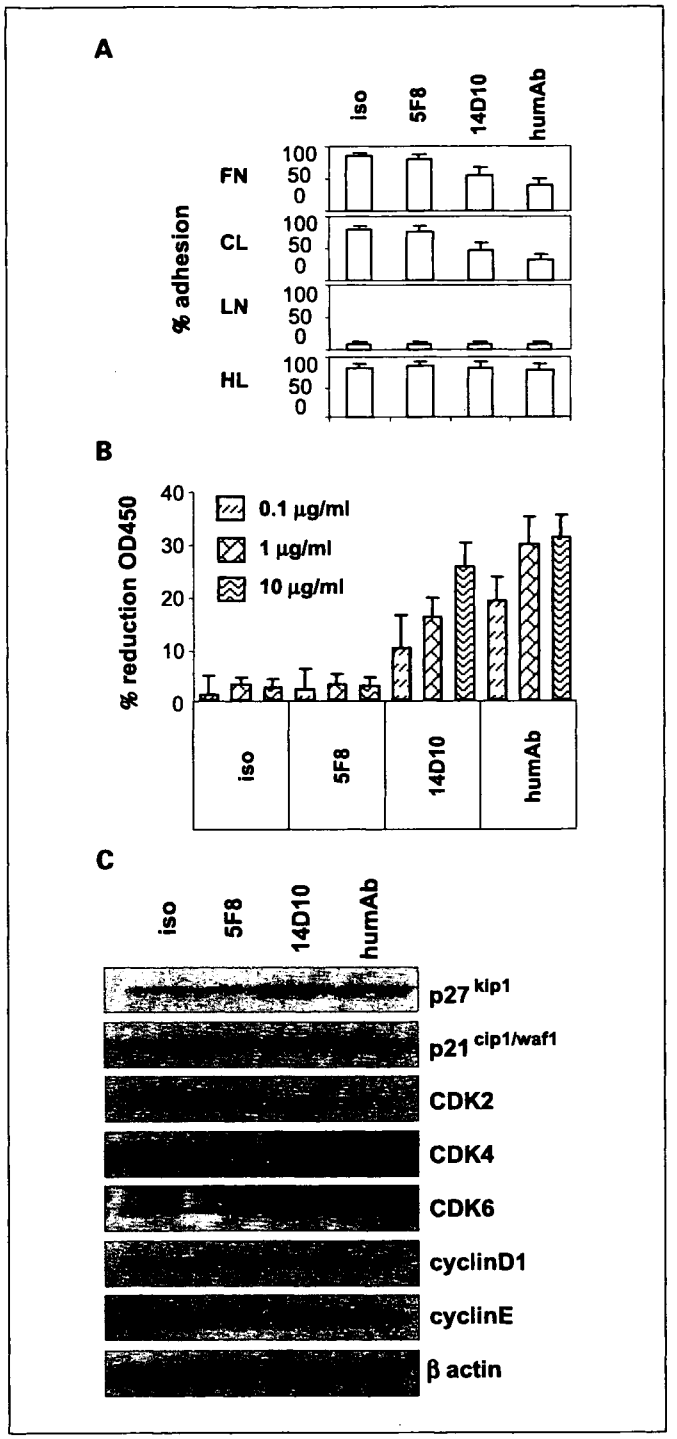


Fig. 2. Inhibitory effect of anti-CD26 mAbs on malignant mesothelioma proliferation. *A*, effect of anti-CD26 mAb on cell adhesion to ECM. JMN cells treated with isotype-matched control mAb (*iso*), 5F8, 14D10, or humanized anti-CD26 mAb (*humAb*) were plated onto 60-mm dishes ($2 \times 10^6$ cells per dish) coated with fibronectin, collagen I, laminin, or hyaluronan and cultured for 18 h. Adhesive cells (%): adhesive cells/adhesive cells + nonadhesive cells. *B*, $5 \times 10^3$ cells per well of JMN were incubated in 96-well plates in the presence of either isotype-matched control mAb, 5F8, 14D10, or humanized anti-CD26 mAb. After 24 h of antibody treatment, water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-methoxy-5-methylphenazinium, was measured at 450 nm using a microplate reader as described in Materials and Methods, and growth inhibitory ratio was calculated as percentage reduction of absorbance 450 nm. *C*, JMN cells were treated with isotype-matched control mAb, 5F8, 14D10, or humanized anti-CD26 mAb. At 18 h after antibody administration, cells were harvested, lysed, and subjected to SDS-PAGE, then probed by specific antibody to $p27^{kip1}$, $p21^{cip1/waf1}$, CDK2, CDK4, CDK6, cyclinD1, cyclinE, and β-actin.

effect than 14D10 (Fig. 2B). Importantly, 14D10 and humanized anti-CD26 mAb induced up-regulation of $p27^{kip1}$ and down-regulation of CDK2. These results suggested that both 14D10 and humanized anti-CD26 mAb dynamically transmit contact inhibition–related growth inhibition via up-regulation of $p27^{kip1}$ and down-regulation of CDK2.

*Humanization of anti-CD26 mAb results in ADCC.* Whereas both 14D10 and humanized anti-CD26 mAb had similar direct effect on cancer cells, our present studies emphasized the different biological effects of humanized anti-CD26 mAb compared with 14D10 through the use of ADCC assay with HuEC. When effector/target (E/T) ratio was held constant at 50, JMN cells treated with humanized anti-CD26 mAb showed specific lysis via ADCC in an antibody dose-dependent manner (Fig. 3A, left). Importantly, JMN cells treated with 14D10 did not show ADCC-specific lysis (Fig. 3A, left), suggesting that humanization of 14D10 to humanized anti-CD26 mAb results in the induction of potent ADCC activity via engagement of the human effector system. Moreover, as seen in Fig. 3A (right), humanized anti-CD26 mAb provoked ADCC-specific lysis in effector-dose–dependent manner. These results were also found when other CD26 positive malignant mesothelioma line besides JMN (NCI-H2452) was used as target cells (Table 2). These data suggested that humanized anti-CD26 mAb possesses a novel biological function other than the direct effect on target cells seen with 14D10, namely ADCC-specific lysis. To better characterize the humanized anti-CD26 mAb–mediated ADCC, apoptosis assays using propidium iodide–annexin V staining and detection of cleaved caspase-3 were used. In these assays, cross-linking method using anti-human IgG, Fcγ fragment specific F(ab')2 fragment of goat, and anti-mouse IgG, Fcγ fragment specific F(ab')2 fragment of goat were used as mimicry of human effectors to humanized anti-CD26 mAb and 14D10, respectively. As seen in Fig. 3B (top three panels), cross-linked humanized anti-CD26 mAb induced late apoptosis, whereas cross-linked 14D10 did not induce late and early apoptosis. Importantly, neither humanized anti-CD26 mAb nor 14D10-induced CDC using human complement (Fig. 3B). To further support these binding, only cross-linked humanized anti-CD26 mAb induced activation of caspase-3 in JMN cells, whereas neither cross-linked 14D10, humanized anti-CD26 mAb plus human complement, and 14D10 plus human complement induced activation of caspase-3 (Fig. 3C). These results therefore indicated that humanized anti-CD26 mAb elicits ADCC-specific lysis but not CDC-specific lysis.

*Humanized anti-CD26 mAb possesses direct* in vivo *antitumor effect on malignant mesothelioma cells.* Because we recently showed that 14D10 exhibits direct *in vivo* antitumor effect on solid tumors (24), we further examined whether humanized anti-CD26 mAb has similar *in vivo* antitumor effect. For this purpose, we used NOD-SCID mice, which lack functional B and T cells as well as most natural killer cell activity (28). To minimize the effect of mouse effector cells, NOD-SCID mice were pretreated by anti–asialo-GM1 polyclonal antisera before being subjected to humanized anti-CD26 mAb functional evaluation. As seen in Fig. 4A and B, humanized anti-CD26 mAb and 14D10 reduced the tumorigenicity of s.c. inoculated JMN, with humanized anti-CD26 mAb being more potent in reducing tumor formation. These observed results suggested that humanized anti-CD26 mAb possesses stronger direct antitumor effect than 14D10. To