Characterization of a novel monoclonal antibody that senses nitric oxide-dependent activation of soluble guanylate cyclase

Shingo Tsuyama[a,]*, Eri Yamazaki[a], Takeshi Tomita[b], Hideshi Ihara[c], Shigeo Takenaka[d], Keiko Kato[e], Shunji Kozaki[a]

[a]*Department of Veterinary Science, Osaka Prefecture University, Sakai, Osaka 599-8231, Japan*
[b]*Institute of Reaction Chemistry, Tohoku University, Katahira, Aoba-ku, Sendai 980-8577, Japan*
[c]*Department of Life Science, Osaka Prefecture University, Sakai, Osaka 599-8231, Japan*
[d]*Laboratory of Nutrition and Food Science, Hagoromo-Gakuen College, Hamadera-minamimachi, Sakai, Osaka 592-8344, Japan*
[e]*Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan*

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Abstract **Two monoclonal antibodies (mAbs) against bovine lung soluble guanylate cyclase (sGC) were prepared and characterized. mAb 3221 recognized both the α- and β-subunits of sGC and had greater binding affinity to the enzyme in the presence of NO. mAb 28131 recognized only the β-subunit and its affinity did not change with NO. Neither mAb cross-reacted with particulate GC. Cultured Purkinje cells from rats were treated with *S*-nitroso-*N*-acetylpenicillamine, an NO donor, and examined by immunocytochemical methods. The immunoreactivity associated with mAb 3221 increased with the cGMP content in a crude extract of cerebellum and the NO_2 generated in the culture medium increased.**

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Key words: Monoclonal antibody; Soluble guanylate cyclase; Nitric oxide; Purkinje cell; Immunoreactivity

1. Introduction

Guanylate cyclase (GC; EC 4.6.1.2), which catalyzes the production of cGMP from GTP, has two forms, particulate and soluble, in mammalian tissues [1]. Soluble GC (sGC) is a receptor of nitric oxide (NO), which stimulates cGMP generation and thereby helps to regulate cell and organ functions involving vasorelaxation, inhibition of platelet aggregation, and neuronal transmission [2]. This enzyme contains one protoheme per heterodimer [3]. Activation of sGC by NO starts with the binding of NO to iron of the prosthetic heme, and proceeds via breaking of the proximal His-Fe bond and the formation of five coordinated nitrosyl-heme complexes [4]. These events in and around the NO-heme complex have been examined in detail by electron paramagnetic resonance (EPR) spectroscopy or resonance Raman spectrophotometry; it seems that they cause conformational changes needed for enzyme activation [5–8]. However, little information is available about whether NO binding causes this critical change, because preparation of the protein crystal for structural analysis has been difficult.

We report here that a newly raised monoclonal antibody (mAb) against sGC has increased affinity to the enzyme in response to NO, suggesting that conformational changes do occur during enzyme activation. This mAb can be used to detect sGC activation immunocytochemically in cultured cells and would be useful for detection of signaling events mediated by NO and sGC.

2. Materials and methods

2.1. Preparation of mAbs to sGC

Soluble GC was purified from bovine lungs by the previously described method [3]. Purified sGC mixed with Ribi adjuvant (Ribi Immunochem., Hamilton, MO, USA) was injected into BALB/c mice, and mAbs were prepared by the method of Harlow and Lane [9]. The reactivity of mAbs 3221 and 28131 was evaluated by Western blotting with sGC purified from bovine lungs and membrane-bound GC partially purified from rat brain [10]. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Dohjin Chemicals (Kumamoto, Japan). A [^{3}H]cGMP immunoassay system and alkaline phosphatase-conjugated anti-mouse IgG were obtained from Amersham Pharmacia. Rhodamine-B-conjugated anti-mouse IgG was purchased from Biosource International (Camarijo, CA, USA). Anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Cappel (Costa Mesa, CA, USA). An mAb to inositol trisphosphate receptors as a marker of Purkinje cells was obtained from Chemicon International (Temecula, CA, USA).

2.2. Effects of mAbs on sGC activity

Purified sGC (100 μg/ml) was incubated for 10 h at 4°C with one of the mAbs at various concentrations. The samples were further incubated for 5 min at 37°C with a mixture with 1 mM GTP, 25 mM triethanolamine-HCl (pH 7.4), 1 mM $MgCl_2$, and 5 mM dithiothreitol (final concentration). The reaction was stopped with the addition of nine volumes of EtOH, and the mixture was centrifuged. The supernatant was dried with a centrifugal vacuum pump, and the dried matter was suspended in a small amount of 25 mM Tris-HCl containing 1 mM EDTA (pH 7.4). The concentration of cGMP was measured by radioimmunoassay. A crude extract of rat cerebellum was obtained by homogenization of cerebella in four volumes of 25 mM triethanolamine-HCl (pH 7.4), containing 50 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The homogenate was centrifuged at $10\,500 \times g$ for 30 min. The supernatant was used as the crude sGC extract.

2.3. Analysis of affinity between mAbs and sGC

For analysis of the interaction between the mAbs and sGC, a Fison IAsys instrument that measures surface plasmon resonance was used. mAb 3221 or 28131 (70 μg/ml) was immobilized on a carboxylmethyl-dextran sensor chip. Purified sGC was added to the sensor chamber in the presence or absence of NO. At times, phosphate-buffered saline containing 0.05% Tween 20 (PBST) was degassed with a vacuum pump and bubbled with NO gas before use. The assay was done at 25°C.

For identification of the epitope specificity of mAb 3221, the subunits of sGC were separated on SDS-PAGE and digested with lysyl-endopeptidase for 12 h at 30°C. The peptides in the digest were purified by HPLC on a micro-ODS column. The column was equilibrated with 0.08% trifluoroacetic acid. Elution was done with a linear gradient of acetonitrile from 0% to 80%. The effluent was monitored

*Corresponding author. Fax: (81) (722) 54-9918.

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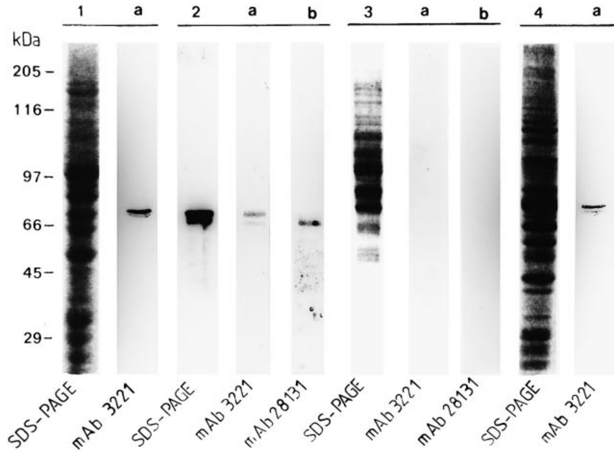

Fig. 1. Western blotting of anti-sGC mAbs 3221 and 28131. Lane 1: SDS-PAGE of a partially purified sGC fraction from bovine lung. Lane 1a: Immunoblots of lane 1 with mAb 3221. Lane 2: SDS-PAGE of purified sGC from bovine lung. Lane 2a: Immunoblots of lane 2 with mAb 3221. Lane 2b: Immunoblots of lane 2 with mAb 28131. Lane 3: SDS-PAGE of partially purified membrane-bound GC from rat cerebellum. Lane 3a: Immunoblots of lane 3 with mAb 3221. Lane 3b: Immunoblots of lane 3 with mAb 28131. Lane 4: SDS-PAGE of partially purified sGC fraction from rat cerebellum. Lane 4a: Immunoblots of lane 4 with mAb 3221.

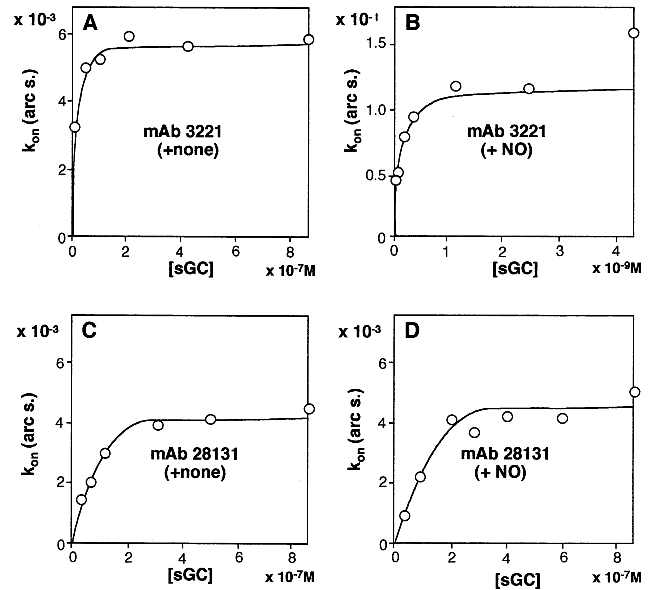

Fig. 2. Analysis of the interaction of anti-sGC mAbs 3221 and 28131 with sGC in the presence and absence of NO. An mAb (70 μg/ml) was immobilized on a carboxylmethyl-dextran sensor chip. Analysis was at 25°C. At times, before use of PBST, it was bubbled for 5 s with NO gas (<0.1 mM). A: mAb 3221 was used, and sGC was added in untreated PBST. B: mAb 3221 was used, and sGC was added in treated PBST. C: mAb 28131 was used, and sGC was added in untreated PBST. D: mAb 28131 was used, and sGC was added in treated PBST.

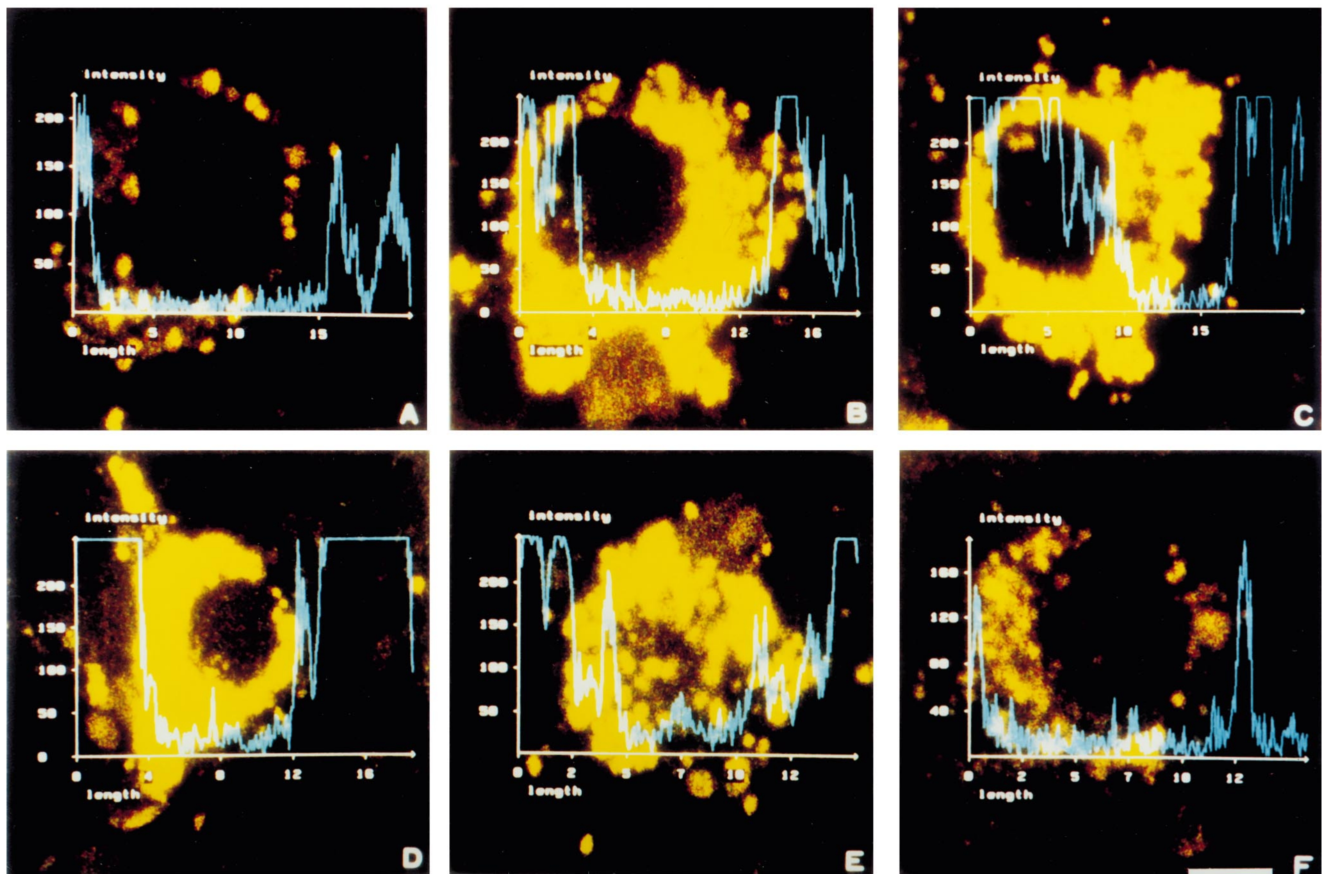

Fig. 3. Fluorescence intensity of soluble GC in Purkinje cells with and without SNAP. SNAP was added in a concentration of 10 μM to the culture medium, incubated, and the cells were fixed with formaldehyde. The cells were stained with mAb 3221 and rhodamine-conjugated anti-mouse IgG. The inset graphs show fluorescence intensity. A, 0 min; B, 5 min; C, 15 min; D, 30 min; E, 45 min; F, 60 min. Bar, 5 μm.

at 210 nm and fractions were collected. The fractions were blotted onto a PVDF membrane and stained with mAb 3221 and peroxidase-conjugated anti-mouse IgG. The fraction reacting with mAb 3221 was analyzed with a protein sequencer (Applied Biosystems, 491).

2.4. Rat cerebellum cell culture

Cerebella were isolated from ten 7-day-old rats. Methods for the culture of cerebellar cells and for identification of these cells were those of Kawashima et al. [11]. Cells were cultured for 7 days at 37°C in an atmosphere of 95% air and 5% CO_2. A portion of the culture medium was incubated in the presence or absence of 10 μM SNAP for up to 1 h at 37°C in a culture chamber. The cells were harvested, fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 10 min, washed three times with PBS, treated with 0.05% Triton X-100 for 10 min, and washed three times with PBS. The mAbs against sGC (7 μg/ml) and two secondary antibodies were incubated separately with each sample. The secondary mAbs were rhodamine-B-conjugated anti-mouse IgG and FITC-conjugated anti-mouse IgG; they were mixed with the cells, which were kept for 1 h at room temperature. The cells were examined by confocal laser microscopy (Leize, CLSM). The fluorescent patches were measured with the pinhole, excitation, and extinction voltages of the confocal laser microscope fixed at 85, 560, and 650 mV, respectively.

We examined changes with time in the concentrations of cGMP of a crude cerebellum extract and of NO_2 in the culture medium after the addition of SNAP with the same culture system. The NO_2 concentration was assayed by a method described previously for diaminonaphthalene [12].

3. Results

3.1. Characteristics of mAbs against sGC

The mAbs, 3221 and 28131, were both κ chains of IgG_1. Western blotting showed that mAb 3221 reacted with both subunits of sGC purified from bovine lungs. However, mAb 28131 bound only with β-subunits, recognized as the band with lower molecular weight. Neither of the mAbs cross-reacted with a partially purified membrane-bound GC fraction with the same enzymatic activity (2 nmol of cGMP/min/mg protein) as GC. mAb 3221 also recognized two proteins in the extract of rat cerebellum, the molecular weights of which were estimated to be 69 000 and 74 000 (Fig. 1). Neither mAb 3221 nor 28131 inhibited sGC.

Results of the interaction between the two mAbs and sGC under various conditions are shown in Fig. 2. With mAb 3221 and PBST not treated with NO, $K_d = 1.12 \pm 0.05 \times 10^{-6}$ M and $B_{max} = 0.0060 \pm 0.0007$ arc s for sGC. When PBST was treated with NO, K_d and B_{max} were $1.22 \pm 0.01 \times 10^{-8}$ M and 0.1265 ± 0.0022 arc s, respectively. When mAb 28131 was used, these values did not depend on the presence of NO. The K_d was $2.42 \pm 0.01 \times 10^{-6}$ M and $B_{max} = 0.0041 \pm 0.0005$ arc s.

mAb 3221 recognized the regulatory domain from Ser-361 to Lys-368 in the α-subunit and from Gly-128 to Lys-151 in the β-subunit.

3.2. Effects of SNAP on immunofluorescence of sGC

When a mAb to inositol trisphosphate receptors was used for the identification of cells, sGC was detected in Purkinje cells when mAb 3221 or 28131 was used; the enzyme was seen as fluorescent patches in the cytoplasm (Fig. 3). When SNAP was present, the immunofluorescence associated with mAb 3221 was greater than when SNAP was not present. The number of fluorescent patches as well as the fluorescence intensity of individual patches increased with time after the addition of SNAP, peaking at 30 min, and then decreasing.

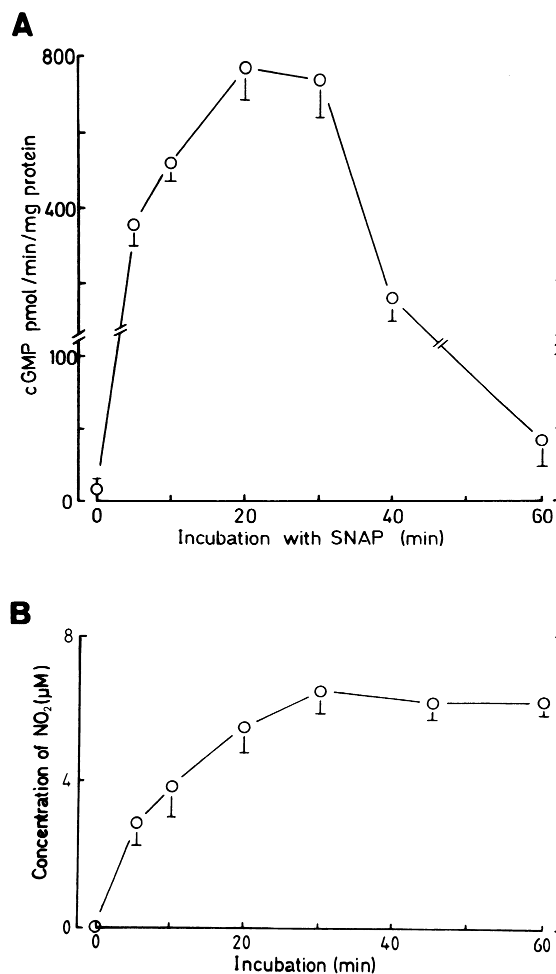

Fig. 4. Effects of SNAP on cGMP production catalyzed by sGC and NO_2 concentrations. A: Changes in cGMP after the addition of SNAP. A crude extract of rat cerebella was incubated for the times shown with 10 μM SNAP at 37°C. After incubation, the mixture was incubated for 5 min at 37°C with 1 mM GTP. Vertical bars show the means of three experiments with the S.D. B: Changes in the concentration of NO_2 after the addition of SNAP to the culture medium. After addition of SNAP (to 10 μM), the NO_2 concentration in the culture medium was assayed. Vertical bars indicate the means of three experiments with the S.D.

The immunofluorescence of sGC did not change after addition of SNAP when mAb 28131 was used (data not shown).

After the addition of SNAP, the cGMP concentration changed with time in a pattern similar to changes in fluorescence intensity (Fig. 4A). NO_2 in the culture medium also increased after SNAP was added, with a maximum at about 30 min (Fig. 4B).

4. Discussion

mAb 3221 recognized changes in sGC caused by NO. With NO, the binding affinity of the mAb was more than 100-fold that without NO. mAb 3221 recognized both α- and β-subunits of the enzyme, but reacted only weakly with the β-subunit (Fig. 1). Perhaps, the mAb 3221 recognized the α-subunit immediately but recognized the β-subunit only after NO-heme binding in the β-subunit. The exact epitope involved in binding with mAb 3221 was not identified, because the crystal structure of the enzyme is still unknown, but our results, in-

cluding the recognition by the mAb of both subunits of the enzyme, suggested that binding with NO caused a conformational change that altered the structural relationship between the two subunits of the enzyme, thereby increasing binding affinity. That the other mAb, which recognized one subunit, did not change in its affinity when NO was present is consistent with this explanation. These new mAbs could be useful for the investigation of the relationship between the enzyme structure and function, when the enzyme becomes available in crystalline form.

Another possible use of mAb 3221 is for detection of sGC activation when NO is added to the culture. The mAb could show where NO-dependent signaling is occurring in the cell. Techniques for demonstration of the generation of NO are already available. Nitrate and nitrite in culture medium can be assayed [12–15], and measurement of cGMP in cells gives semiquantitative information about NO generation in various experimental systems [16–18]. However, these methods give only indirect evidence of such generation. EPR spectroscopy gives direct evidence of the presence of NO, which becomes detectable as an adduct of an NO-trapping reagent. NO-associated adducts can now be made visible in living experimental animals [19,20], but it has not been technically possible to explore the microtopography and changes with time in NO generation in the cell. Our study showed that mAb 3221 can detect conformational changes in sGC during formation of NO-sGC complexes, and thus detect the generation of NO. The mAb might be useful in tissue and organ histochemical studies.

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