FcαRI/CD89 Circulates in Human Serum Covalently Linked to IgA in a Polymeric State

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The FcR for IgA CD89/FcαRI, is a type I receptor glycoprotein, expressed on myeloid cells, with important immune effector functions. In vitro CD89 can be released from CD89-expressing cells upon activation. Little information is available on the existence of this soluble molecule in vivo. Using specific and sensitive ELISA techniques (detection limit 50 pg/ml), we were not able to detect circulating CD89 in human sera. However, using Western blotting, a 30-kDa soluble CD89 molecule was demonstrated in both serum and plasma. Moreover, using a specific semiquantitative dot-blot system, we found CD89 in all human sera tested (mean concentration 1900 ng/ml). Size fractionation of human serum using gel filtration chromatography showed that the CD89 molecule was predominantly present in larger molecular mass fractions. Direct complexes between IgA and CD89 were demonstrated by anti-IgA affinity purification, and when analyzed under nonreducing conditions appeared to be covalently linked. Size fractionation of affinity-purified IgA showed the presence of soluble CD89 only in the high molecular mass fractions of IgA, but not in monomeric IgA. High molecular mass complexes of CD89-IgA could be distinguished from J chain containing dimeric IgA. These data show that CD89 circulates in complex with IgA, and suggest that CD89 might contribute to the formation of polymeric serum IgA. The Journal of Immunology, 2002, 168: 1252–1258.

Receptors for the Fc portions of Igs have been identified on a variety of cell types within the immune system and provide a crucial link between the humoral and cellular arm of the immune system. In humans, IgA is the predominant isotype produced (≈66 mg/kg/day), and plays a critical role in protecting the host against environmental pathogens and Ags encountered at mucosal surfaces (1). At present, a single specific IgA-FcR has been cloned and characterized.

FcαRI/CD89 is a type I transmembrane glycoprotein expressed at the surface of myeloid cells and member of the Ig superfamily (2, 3). CD89 binds both IgA1 and IgA2 with similar affinity (Kd ~ 10^(-6) M^(-1)). The site of interaction between CD89 and IgA was identified in the first extracellular domain of CD89 (4, 5) and the Ca2/Ca3 junction of IgA (6). CD89 is constitutively expressed as a 50- to 70-kDa protein on neutrophils and monocytes/macrophages, or as a 70- to 100-kDa glycoprotein on eosinophils due to increased glycosylation (7). CD89 is also expressed on Kupffer cells in the liver, where it was suggested to provide a second line of defense (8). At the cell surface, CD89 is associated with the FcR γ-chain through the charged argin residue within its transmembrane domain (9, 10). Although it is still controversial whether this association is necessary for surface expression (11, 12), it is critically important for signal transduction via CD89. Cross-linking of CD89 on myeloid cells triggers diverse processes including phagocytosis, superoxide generation, Ab-dependent cellular cytotoxicity, and release of inflammatory mediators (8, 13).

Release of soluble receptors is a universal mechanism of receptor regulation and soluble forms of FcR for IgG (FcγRII/CD132 and FcγRIII/CD16) and IgE (FcεRII/CD23) have been demonstrated both in vitro and in vivo (14). The existence of circulating IgA-binding factors has been described (15), but their molecular structure has remained unclear.

Recently, we showed that upon activation, a soluble form of CD89 is released from the surface of monocytes and monocytic cell lines (16). The molecule was identified as a 30-kDa glycoprotein, which has still retained the capacity to bind IgA. At present, little information is available concerning the presence of soluble FcαRI/CD89 in biological fluids. In the present study, we show by Western blot and semiquantitative dot-blot that all sera tested from healthy volunteers contain a soluble (s)3 CD89 molecule. However, the molecule circulates in a complex covalently linked with IgA in the high molecular mass fractions of serum IgA.

Materials and Methods

ELISA for sCD89

CD89 in serum was detected by ELISA as described previously (16). In short, rabbit anti-CD89 IgG (2 μg/ml) was coated to ELISA plates by overnight incubation at room temperature in coating buffer (0.1 M NaHCO3/Na2CO3, pH 9.6). Alternatively, mAbs against the extracellular domain 1 (2D11, 2H8) or extracellular domain 2 (7D7, A77) domain of CD89 (5, 17) were used as catching Ab (2 μg/ml in coating buffer). The wells were washed three times using washing buffer (PBS, 0.02% Tween 20) and samples were applied. Purified recombinant sCD89 was used as a standard curve and serum samples were tested in serial dilutions (1/2–1/100). All dilutions and subsequent Ab steps were performed in ELISA buffer (PBS, 0.02% Tween 20, 1% FCS). Following 1 h incubation at 37°C, wells were washed as above and incubated with digoxigenin-conjugated

Received for publication May 7, 2001. Accepted for publication December 4, 2001.

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1 This research was financially supported by a grant from the Netherlands Organization for Scientific Research (901-12-214). C.v.K. is a fellow of the Royal Dutch Academy of Science.

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3 Abbreviations used in this paper: s, soluble; PVDF, polyvinylidene difluoride; plgR, polymeric IgR; slgA, secretory IgA; NHS, normal human serum.
rabbit F(ab’)_2, anti-CD89 (1 μg/ml), followed by HRP-conjugated F(ab’)_2 anti-digoxigenin (1/5000; Boehringer Mannheim, Indianapolis, IN; both for 1 h at 37°C and washed in between as above). The OD at 415 was measured after addition of ABTS/H_2O_2 as substrate.

**Isolation of sCD89**

As a positive control, sCD89 protein was isolated from culture supernatant of PMA/ionomycin (10 ng/ml and 1 μg/ml, respectively) stimulated U937 cells using an affinity column of human IgA isolated from normal serum, as described before (16). The purity of the preparations was checked on a 10% SDS-PAGE and a single band of 30 kDa in size was detected by Coomassie brilliant blue staining. Recombinant sCD89 (18) was produced in Chinese hamster ovary cells and purified similar to the natural sCD89 molecule (16).

**Western blot analysis**

Purified or recombinant sCD89, as well as serum and plasma samples obtained from healthy individuals, were separated on 10% SDS polyacrylamide gels under reducing conditions, and blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). In all cases, 30 μl sample was mixed with 15 μl 3× sample buffer (Bio-Rad Laboratories, Hercules, CA) and boiled for 5 min. For comparison, several formulations of prestained wide range molecular mass markers (Bio-Rad) were run on the same gels. Using standard Western blotting protocols, different forms of CD89 were detected with rabbit IgG anti-CD89 (10 μg/ml; Ref. 16). After incubation and washing followed by incubation with HRP-conjugated swine anti-rabbit-IgG (1/50,000; DAKO, Glostrup, Denmark), signals were visualized using Super Signal Chemiluminescence substrate, according to manufacturer’s instructions (Pierce, Rockford, IL).

Alternatively, samples were analyzed under nonreducing conditions on either 10 or 6% SDS-PAGE gels, or precast 4–12% gels (Bio-Rad). They were analyzed by Western blotting using the monoclonal anti-CD89 Ab 7D7 (IgG1) at 10 μg/ml, followed by HRP-conjugated goat anti-mouse-Ig (DAKO) and chemiluminescence as described above.

IgA was detected in Western blot using the monoclonal anti-IgA Ab 4E8 (IgG1) at 2 μg/ml (19). J chain was detected in Western blot using a J chain-specific rabbit polyclonal antiserum (20, 21).

**Detection of sCD89 by dot-blot technique**

Sera were diluted 1/100, 1/1,000, and 1/10,000 in PBS and blotted onto a PVDF membrane using a special dot-blot device (Bio-Rad), according to manufacturer’s instructions. Alternatively, fractions obtained from gel filtration columns were blotted. Membranes were developed for CD89 reactivity with the polyclonal anti-CD89 antiserum as described for Western blotting. As a standard, we included purified sCD89 molecule derived from U937 cells on every blot, in a concentration range starting from 10 ng/ml. For specificity control, the anti-CD89 antiserum was preincubated with 25 μg/ml recombinant sCD89 produced in Chinese hamster ovary cells, which prevented specific binding. Densitometry was performed using the Eagle-Eye software package (version 3.2; Stratagene, San Diego, CA).

**Fractionation of serum**

Randomly selected sera from healthy controls (n = 6) were separated by size on a 1.5 × 90 cm Sephacryl S300 column (Amersham Pharmacia, Roosendaal, The Netherlands). Briefly, 0.5-ml aliquots of sera were diluted with an equal volume of PBS layered on the column, and fractions of 1.25 ml were collected. The fractions were analyzed for protein content using BCA protein assay (Pierce), and CD89 using ELISA- and CD89-specific bloting.

**IgA affinity purification and fractionation**

A total of 50 μl of serum was diluted with 950 μl of PBS-EDTA, mixed with anti-IgA immunoabsorbent (mAb 4E8-Sepharose, produced in our laboratory), rotated at 4°C overnight, and subsequently poured in a small column. The fall-through was collected, followed by extensive washing with PBS-EDTA. Bound IgA was eluted with 0.1 M glycine-HCl, 0.3 M NaCl (pH 2.8), and neutralized by 15 μl Tris buffer per milliliter.

Purified IgA, or total serum as comparison, were separated by size on a 26/60 HR200 Superdex column (Amersham Pharmacia) and fractions of 2 ml were collected. The fractions were analyzed for protein content using BCA protein assay, total IgA using ELISA (sensitivity 2 ng/ml; Ref. 19), and CD89 using ELISA and CD89-specific bloting.

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**FIGURE 1.** CD89 Western blot of NHS. Four different normal human sera (A–D) were diluted 1/50 and ran under reducing conditions in 10% SDS-PAGE. Proteins were transferred to PVDF membrane and analyzed for CD89 using polyclonal anti-CD89 Abs. For specificity control, the antiserum was preincubated with rCD89 (25 μg/ml; right panel). As a reference for the soluble CD89 molecule previously described, supernatant of activated U937 cells was run in parallel.

**FIGURE 2.** CD89 in serum cannot be detected by ELISA. Serum or plasma samples of a representative individual were tested in different CD89 specific ELISAs using either polyclonal (rabbit anti-CD89) or mAbs (7D7, A77, 2D11, and 2H8) as catching Ab. Control rabbit Ig or an irrelevant IgG1 Ab were used as control. No reactivity is observed in either serum or plasma (data shown 1/50 dilution), in supernatant (1/2 dilution) of nonactivated U937. As a positive control, all ELISA systems were able to detect CD89 in supernatant (1/10 dilution) of PMA/ionomycin-activated U937.
Results
A 30-kDa form of CD89 is present in both plasma and serum as detected by Western blotting

To investigate the presence of sCD89 molecules in circulation, Western blotting was performed on samples separated by SDS-PAGE under reducing conditions, as described before. In both serum (Fig. 1) and plasma, a 30-kDa CD89 molecule could be visualized. This band has a molecular mass which corresponds with the sCD89 molecule released by U937 cells after activation with PMA/ionomycin (Fig. 1, lane 1; Ref. 16). Preincubation of the detecting Ab with rCD89 (25 µg/ml) completely abrogated the signal, proving specificity of this Western blot detection (Fig. 1, lanes 6–9).

sCD89 cannot be detected in human plasma or serum using specific ELISA

To quantify the amount of circulating CD89 in human serum, we used different CD89-specific ELISAs based on polyclonal reagents of rabbit origin (16), or using mAbs directed against the extracellular domains 1 or 2 of CD89. We tested serial dilutions (1/2–1/100) of serum or plasma from 48 healthy volunteers. None of the samples showed reactivity in any of these CD89-specific ELISAs (Figs. 2 and 3B). In contrast, sCD89 present in supernatant of PMA/ionomycin-activated U937 cells could be detected with both polyclonal and mAbs as catching Ab (Fig. 2). Moreover, the same ELISAs were able to detect recombinant sCD89 added to human serum (data not shown).

Measurement of CD89 levels using dot blot

Because Western blotting revealed a single 30-kDa band which was completely inhibited by preincubation with recombinant

FIGURE 3. Detection of CD89 in NHS. A, Using the same reagents as described for Western blot, a dot-bl ot system was developed. Both NHS, as well as a standard preparation of sCD89 purified from activated U937 showed a dose-dependent reactivity. The signal was prevented by preincubation of the detecting antiserum with rsCD89 (25 µg/ml). B, A total of 48 normal human sera were tested in parallel in dot-blot (left) and ELISA (right). The amount of reactivity was related to a standard preparation of purified sCD89. The dotted line represents the detection limit of both assays, being 1 and 50 pg/ml, respectively.

FIGURE 4. sCD89 circulates as a high molecular mass protein. A, Sample of NHS is separated by size on a S-300 column followed by analysis of protein content (□) as well as CD89 reactivity using dot-blot (○). The molecular mass of the qualified column are shown on top of the figure. One representative fractionation out of six is shown. B, Starting material (NHS; 1/50 dilution), high (110, 130, and 150 ml) and low (215 and 200 ml) volume fractions (30 µl) were analyzed by Western blot as described in Fig. 1. C, Serial dilutions of NHS (1/320, 1/160, 1/80, and 1/40) were run in 10% SDS-PAGE under reducing (left panel) and nonreducing (right panel) conditions. Next, proteins were transferred to PVDF membranes and analyzed for CD89 using the monoclonal anti-CD89 7D7.
sCD89, we have set up a dot blot system specific for CD89, using the same reagents. Using purified sCD89 from PMA/ionomycin activated-U937 as a standard, dose-dependent increase in dot blot reactivity was observed (Fig. 3A). A similar dose-response curve was obtained when applying different dilutions of normal human serum (NHS). In both cases, all reactivity could be inhibited by preincubation of the antisera with recombinant sCD89 (25 μg/ml; Fig. 3A).

Using a dose response of purified sCD89 on every dot blot, the amount of sCD89 in human serum was quantified using densitometry. In the same panel of serum samples of healthy controls (n = 48) which were negative in ELISA (<50 pg/ml), we found the presence of CD89 in all cases tested. The estimated mean value of CD89 in these sera was 1900 ng/ml (Fig. 3B).

**CD89 circulates in a high molecular mass complex**

To study the molecular nature of CD89 in human serum, we fractionated sera using a Sephacryl S300 column and tested the fractions for CD89. Using the dot-blot technique, we could only detect CD89 in the high molecular mass range above 150 kDa, fraction volumes from 105 to 155 ml, but not in fraction volumes 205–210 ml, where 30-kDa proteins are expected (Fig. 4A). None of the fractions showed reactivity in the CD89 ELISA (data not shown). A similar profile was found with six independent sera.

Analysis of these samples in Western blot showed, under reducing conditions, the presence of the same 30-kDa molecule in the high molecular mass fractions (>150 kDa) as observed in total serum (Fig. 4B). The same 30-kDa band was observed when the Western blot was performed with the monoclonal anti-CD89 Ab 7D7 instead of the polyclonal antiserum (Fig. 4C). Interestingly, samples run under nonreducing SDS-PAGE conditions revealed CD89 reactivity around 180 kDa, when Western blots were stained with this 7D7 mAb.

**Affinity purification of IgA-CD89 complexes**

Since we previously showed that sCD89 was still able to bind IgA (16), specific interaction with IgA was the prime candidate to explain the high molecular mass appearance of CD89. Therefore, IgA was purified from serum samples using anti-IgA affinity purification. IgA was purified from human serum with a recovery of 95%, and was pure as judged by Coomassie blue staining (data not shown). Size fractionation of this purified IgA showed a complete overlap of the IgA profile and the protein content (Fig. 5A, right panel). The size distribution of purified IgA was comparable to total serum (Fig. 5A, left panel).

Purified IgA was analyzed in Western under nonreducing conditions. Both starting material and the eluate from the affinity column showed strong high molecular mass staining for CD89 (Fig. 5B). These data confirm the presence of IgA-CD89 complexes.

**CD89 is exclusively present in circulating dimeric and polymeric IgA**

In circulation, IgA is present in different molecular sizes being either monomeric, dimeric, or polymeric in nature. To determine whether sCD89 preferentially associates with certain IgA types, IgA was isolated from serum using the above described affinity purification, followed by size fractionation. Measurement of IgA in these fractions using ELISA showed the typical distribution of IgA, with the majority being monomeric (Fig. 6A). When the same fractions were analyzed by CD89-specific dot blot, reactivity was most pronounced for dimeric and polymeric IgA, and almost absent from the monomeric fraction.
Different fractions of IgA were applied in nonreducing SDS-PAGE on 4–15% gradient gels, blotted, and Western was developed for IgA and CD89. Staining for IgA showed a major band around 180 kDa in the monomeric fraction (Fig. 6B). The dimeric IgA fraction consisted of two major bands, one representing dimeric IgA and one migrating slightly faster as monomeric IgA. Finally, IgA circulating in a higher molecular mass form (polymeric) showed a similar pattern as dimeric IgA fractions.

Next, the same samples were analyzed for CD89. The reactivity for CD89 was found at the height of the lower molecular mass IgA molecule, but exclusively in these fractions which appear as higher molecular mass in gel filtration (Fig. 6B).

IgA-CD89 and IgA-J chain complexes can be distinguished in dimeric serum IgA

To further investigate the molecular nature of the two differently sized IgA molecules in the dimeric IgA fraction, we performed Western blotting with a specific anti-J chain antisemur (20, 21). Anti-J chain specifically detected the upper 350-kDa dimeric IgA band in Western blot, but not the CD89-IgA complex (Fig. 7). IgA, truly monomeric both in gel filtration and Western, was completely negative for J chain or for CD89, even when larger amounts of IgA were loaded (Fig. 7). Therefore, high molecular mass serum IgA might be either formed by covalently linked, J chain containing, dimeric IgA, or by multimerization of covalent IgA-CD89 complexes.

Discussion

In the present study, we demonstrate the presence of a soluble form of the FcR for IgA (CD89) in all human sera tested. Under reducing conditions, the molecule was identified as a 30-kDa molecule, comparable with the soluble molecule released in vitro from the surface of myeloid cells (16). However, it was found that the molecule circulates in a high molecular mass form in complex covalently linked to IgA. In gel filtration, all IgA-CD89 complexes were found in the higher molecular mass fractions, suggesting that interaction of IgA with CD89 facilitates the formation of polymeric serum IgA.

Covalent linkage between IgA and the soluble form of a cell surface receptor is not unique for the IgA-CD89 complex, as described in the present paper. Dimeric IgA containing J chain uses binding to the polymeric IgR (pIgR) for transcytosis from the basal to the apical site of epithelial cells in the mucosa (22). During a late phase of the transcytosis process, IgA becomes covalently bound to pIgR.
linked to the pIgR (23, 24). At the apical membrane, this receptor is proteolytically cleaved, and IgA is released as secretory IgA (sIgA), retaining the so-called secretory component (25). Mice lacking the J chain are deficient in the formation of sIgA (26), and have a phenotype comparable to pIgR-deficient mice (27). Interestingly, J chain knockout mice do show the formation of higher molecular mass IgA in serum, confirming a multimerization of IgA in the absence of J chain. This might suggest the involvement of other molecules, but since a murine CD89 homolog has not yet been identified, in these cases the role of CD89 cannot be addressed.

Our data show that J chain-containing dimeric IgA is completely negative for CD89. Therefore, it can be hypothesized that J chain and CD89 are competing for the same cytosome. In our Western blot experiments, we could clearly distinguish CD89-IgA complexes and J chain-containing dimeric IgA. However, this does not mean that dimeric IgA cannot interact with cell surface CD89, as demonstrated before. At the moment, we favor the hypothesis that the site of covalent linkage can occur at a different location from the initial binding site, in line with dIgA-pIgR interaction and the generation of sIgA (28).

Our study suggested a discrepancy between detection of circulating CD89 in ELISA or dot-blot. However, this difference can be explained by the observed covalent interaction between CD89 and IgA. CD89 consists of two Ig domains, both formed by a single intradomain disulfide bond (2). Therefore, the covalent linkage to IgA will have dramatic structural consequences. This is in contrast to the formation of sIgA, where the fifth Ig domain, which becomes covalently linked to IgA, contains three intradomain disulfide bonds, allowing one bond to be used for interaction with IgA (28). This most likely explains why CD89-IgA complexes cannot be detected by ELISA, even when the amount of CD89 estimated by dot-blot is many orders higher than the detection limit of the ELISA.

An important open question is the mechanism of complex formation. In analogy with the pIgR system, it is most likely that reshuffling of cysteine bonds takes place in an intracellular compartment. Previously, we have shown that the common γ-chain is necessary for the generation of the 30-kDa CD89 molecule and that IgA is a potent inducer of shedding in monocytic cells, but not neutrophils (16). Others have suggested that CD89 can exist both in the absence or presence of the γ-chain (12). In the absence of γ-chain, IgA which binds to CD89 is internalized and localizes mainly in early endosomes. However, in the presence of γ-chain, CD89 and bound ligands are internalized and transported into an endolysosomal compartment (12). Especially in cells like monocytes, such a compartment is known to have special features (29). Therefore, this could be a potential mechanism by which monomeric IgA becomes covalently linked with CD89. Although induction of signal transduction via CD89 is more efficient with high molecular mass IgA complexes (30), Biacore experiments have clearly demonstrated that monomeric IgA can also efficiently interact with CD89 (31).

Recently, complexes of CD89 and IgA have been described in serum of patients with IgA nephropathy (32). In this study, sCD89 could be detected by ELISA, although only after polyethylene glycol precipitation. In contrast to our previous data (16), the sCD89 molecule described was spontaneously released from the surface of monocytes in vitro. This shedding was independent of signaling via the γ-chain, and the molecule appeared to have a different molecular mass (32). Moreover, sCD89 was only detected in patients with IgA nephropathy, but not in healthy controls. In preliminary experiments, we have observed that covalent IgA-CD89 complexes are equally present in both groups (P. van der Boog, manuscript in preparation). These data strongly suggest that CD89 can circulate in different molecular forms, either low concentrations of free or IgA-bound CD89 (ELISA detectable) and high amounts of covalent CD89-IgA complexes.

The pathophysiological role of IgA-CD89 complexes is currently unknown. Soluble forms of other FcR, specific for IgG or IgE, have been extensively described in the past. Circulating molecules with IgE-binding capacity (IgE-binding factors) were identified as the soluble form of FcεRII (sCD23; Refs. 33 and 34), and play an important role in the regulation of IgE production. In NHS, low levels of FcγRII (sCD32; ~10 ng/ml) and higher levels of sFcγRII (sCD16; ~2.5 µg/ml) have been demonstrated (14). These levels of sCD16 are in the same range as what we observed for circulating CD89. Release of soluble receptors will terminate the ongoing signaling process and might represent a mechanism to prevent prolonged activation. Moreover, release of soluble receptors that still retain their ligand binding capacity will also help to terminate the signaling process. The latter is most likely not the case for the circulating CD89 described in the present paper, since it is already associated with IgA, and the structure of the protein is most likely disturbed. Alternatively, facilitation of the formation of high molecular mass IgA by sCD89 might result in more efficient binding and cross-linking of the cell surface CD89 receptor. Therefore, it will be important to investigate and compare the effector functions of IgA and IgA complexed with CD89, including binding to other receptors, clearance in vivo, or resistance to proteolysis. In conclusion, we have demonstrated that a part of serum IgA consists of IgA-CD89 complexes. CD89 and monomeric IgA form a covalently linked complex, which seems to facilitate multimerization to polymeric serum IgA and circulates in high molecular mass fractions. We have estimated that up to 5–10% of serum IgA might be complexed with CD89, but more detailed studies are needed to confirm this. By Western blot, the CD89-IgA complex can be distinguished from J chain-containing dimeric IgA. It will be important to determine the effector functions of these CD89-IgA complexes, which might add another complexity to the role of IgA in both mucosal and systemic immunity.
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