Signaling through CD40 Rescues IgE but Not IgG or IgA Secretion in X-linked Immunodeficiency with Hyper-IgM

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Abstract

The ligand for CD40 (CD40L) is a membrane protein on activated T cells that induces B cell proliferation and differentiation. Several mutations of the CD40L gene were reported responsible for defective class switching of B cells in an X-linked immunodeficiency with hyper IgM (X-HIM).

We studied four affected males from three families and found three independent mutations including new mutations of CD40L gene. In every X-HIM patient tested, however, anti-CD40 plus IL-10 did not induce class switching from IgM to IgG or IgA, even in the presence of Staphylococcus aureus Cowan I strain (SAC). CD4+ T cell clones, expressing CD40L on their surface, also did not rescue IgG or IgA induction by X-HIM peripheral blood B cells in vitro. But signaling through CD40 induced both B cell proliferation and IgE secretion when IL-4 was added to the culture.

Taken together, these results show that in vitro signaling through CD40 rescues IgE but not IgG or IgA secretion by peripheral blood X-HIM B cells and suggest that in vivo CD40 and CD40L interaction might be necessary for IgG and IgA differentiation in X-HIM. (*J. Clin. Invest.* 1995. 95:510–514.) Key words: CD40 • IgE • B cell • IgM • immunodeficiency

Introduction

Immunodeficiency with hyper IgM (HIM)[1] is a rare disorder characterized by recurrent bacterial infections associated with very low or absent IgG, IgA, and IgE and normal to increased IgM serum levels (1). Evidence for clinical heterogeneity has been provided by the demonstration that the HIM syndrome can be either occur as a primary or as an acquired disorder (2, 3). Primary HIM is a very rare disease, which accounts for as little as 0.3–2.0% of all primary immunodeficiencies (4).

Several reported pedigrees are consistent with an X-linked inheritance. However, occurrence in females suggest that primary HIM syndrome can also be inherited as an autosomal-dominant transmission (1, 3). Immune abnormalities in HIM syndrome are in most cases restricted to B cells with secretion of IgM only (5, 6). However, some patients have been reported with concomitant cellular immune defects resulting in a combined immune deficiency (7).

The CD40 cell surface antigen on B cells acts as a receptor capable of transmitting a signal (8, 9). The ligand for CD40 is a membrane glycoprotein on activated T cells and the recent isolation and characterization of cDNA clones encoding gp39 have made possible the detailed study of this receptor–ligand pair and it's role in B cell activation (10–12). A series of experiments have revealed that the signals delivered to B cells via CD40 can synergize with other costimulatory signals to drive B cell proliferation and differentiation (13–16). In X-HIM patients, a series of recent papers reported that CD40L expression is incomplete on the surface of activated T cells and the defect is due to several distinct mutations of the gene coding for CD40L (17–20). The resultant failure of CD40 and ligand interaction is suggested responsible for the Ig isotype switching defects. It is also reported that in vitro signaling through CD40 rescues not only IgE but also IgG and IgA production (19, 21).

In the this study, we confirmed independent mutations of the CD40L gene among different X-HIM families. However, signaling through CD40 rescues only IgE production by patient's B cells, suggesting that further B cell defects might reside in this disease.

Methods

Patients. Four male HIM patients from three families participated in this study. Case 1 (3 yr old) and 2 (7 yr old) are brothers. Besides case 1 and 2, their mother and father and healthy brother (5 yr old) also participated in this study. During the study, case 2 died of fungal meningitis at age 7. Case 3 (5 yr old) and 4 (6 yr old) are HIM patients from different families and all patients receive transfusion therapy of gamma globulin. Other X-HIM related patients, such as female HIM and common variable immunodeficiency (CVI) patients also participated in this study. A female HIM and two male type I CVI (22) patients whose B cells differentiate to only IgM secretion were examined.

Amino acid sequence analysis of genes coding for CD40L. IL-2–dependent T cell lines from patients and EB virus transformed B cell lines were established by methods described elsewhere (23). Total RNA was extracted and reverse transcription of CD40L mRNA to cDNA was done according to standard methods using the primer for CD40L (19).

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1. *Abbreviations used in this paper:* CD40L, ligand for CD40; CVI, common variable immunodeficiency; HIM, hyper IgM; SAC, Staphylococcus aureus Cowan I strain; Tc, T cell clone; TdR, thymidine; X-HIM, X-linked immunodeficiency with HIM.

The resulting product, which encompasses the entire coding region of CD40L (10), was cloned into Bluescript SK(+) and SK(−) (Stratagene, La Jolla, CA) using EcoRI–Sal sites incorporated in the two primers. To eliminate potential sequence artifacts caused by PCR, reverse transcription and PCR, and subsequent cloning of CD40L cDNA from activated cells of each patient, were done more than twice in independent experiments. For sequencing, at least 40 single clones were picked in every independent cloning experiment, pooled, and used for generation of single-stranded DNA. Entire CD40L coding regions were then sequenced manually.

Preparation of cells. Peripheral blood mononuclear cells (MNC) were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation of heparinized blood and were washed three times with RPMI 1640 (Nikken Bio Medical, Kyoto, Japan), as described previously (24). In some experiments, T cells were removed by twice rosetting the cells with aminoethylisothiuronium bromide treated sheep blood cells and monocytes were partially depleted by a plastic adherent method (24). The remaining cell population, referred to as B cells, consisted of > 96% B cells (CD 20) and < 2% T cells (CD3).

Reagents for B cell stimulation. Usually 5C3 anti-CD40 mAb (Pharmingen, San Diego, CA) was used at concentration 1 μg/ml which induced the maximum Ig secretion in human B cells. In some experiments, another anti-CD40 mAb (B-B20, 1 μg/ml, Serotec, Kidlington, England) was used.

SAC, originally obtained from the National Collection of Type culture (London), was used. The bacteria were killed by incubation in 0.5% formaldehyde for 3 h at room temperature and then heat-killed at 80°C for 5 min (25). The bacteria were harvested and washed three times with sterile saline and resuspended as a 10% stock solution in PBS. SAC was used at concentration 0.001% vol/vol (24).

Induction of immunoglobulin secretion. MNC (10⁵/well) were cultured in 96-well plates (Falcon, Oxnard, CA) in 200 μl of RPMI 1640 supplemented with 10% FCS (Sterile Systems, Logan, UT), 2 mM glutamine. IL-10 (100 ng/ml; Genzyme Corp., Boston, MA) and IL-4 (Genzyme, 100 U/ml) were added to the culture at the first day. Supernatants were harvested on day 14 and IgE concentration was determined by previously described enzyme linked immuno-sorbent assay (ELISA, 26) with some modification. In brief, microplates (Nunc-Immunoplate, Maxisorp, Denmark) were coated with 100 μl of anti–human IgE antibody (6-90, 1 μg/ml) over night at 4°C. After blocking with 0.1% of BSA, a total volume (100 μl) of diluted supernatant was assayed. After 2 h incubation at room temperature, alkaline phosphatase labeled anti-human IgE mAb (8–73) was added and incubated for 2 h at room temperature. After washing the wells four times, 100 μl of p-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO) solution was added to each well and absorbance at 405 nm was examined with an ELISA reader (ImmunoReader Nr-2000, InterMed). Purified IgE from IgE myeloma cell line U266 was used as the standard. IgM, IgG, and IgA secretion was also examined by isotype specific ELISA (Tago Inc., Burlingame, CA) as reported previously (24).

Preparation of CD4+ T cell clones and cell culture. T cells separated by E rosette formation were stimulated with PHA (10 μg/ml; Difco Laboratories Inc., Detroit, MI) and IL-2 (1 U/ml; Takeda, Osaka, Japan) every 4 d. After limiting dilution, CD4+ clones were selected and expanded in Opti-MEM (GIBCO-BRL, Gland Island, NY) liquid medium supplemented with 10% FCS and further stimulated weekly with feeder cells (27). CD4+ T cell clones from a healthy brother express normal CD40L but those from case 1 express a mutation leading to a truncated CD40L (19). CD4+ T cell clones were irradiated at 3000 rad. and 2 × 10⁵ cells were cultured in anti-CD3 (Leu 4, 100 ng/ml; Becton Dickinson, Mountain View, CA) coated microculture plates.

Assay of [³H]Thymidine (TdR) Incorporation. MNC (10⁵/well) were cultured in 96-well, flatbottomed microculture plates (Falcon, Oxnard, LA) for 5 d and each culture was pulsed [³H]TdR (DuPont-New England Nuclear, Boston, MA) for 4 h. Subsequently, incorporation of [³H]TdR was examined by a liquid scintillation counter (24).

Results

Mutations in the coding region of CD40L in HIM patients. RNA from HIM patients was reverse-transcribed into cDNA

CASE 1 and 2 and their family.

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NA 462   TCT GTG TTA CAG TGG GCT GAA GAA    485
AA 136   S   V   L   Q   W   A   E   K      143
                           475 (G→A)

FATHER   --- ---  --- --- --- --- --- ---
MOTHER   --- ---  --- --- --- --- --- ---
         --- ---  --- --- -A- --- --- ---
CASE 1   --- ---  --- --- -A- --- --- ---
BROTHER  --- ---  --- --- --- --- --- ---
CASE 2   --- ---  --- --- -A- --- --- ---
                      TAG (stop codon, 140)

CASE 3
NA 753   CCA GGT GCT TCG GTG TTT GTC AAT    776
AA 233   P   G   A   S   V   F   V   N      240
                           766 (T→A)
CASE 3   --- ---  --- --- -A- --- --- ---
                      GAG(GLU, 237)

CASE 4
NA 684   AAT ACC CAC AGT TCC GCC AAA CCT    707
AA 210   N   T   H   S   S   A   K   P      217
                    * deletion (692 C, 693 A)
CASE 4   --- ---  --* *-- --- --- --- ---
                  G TTC CGC CAA ACC TTG
                  Q   F   R   Q   T   L
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Figure 1. Mutations of CD40L gene in X-HIM Patients. Amino acid sequence analysis of genes coding for CD40L were examined in four male X-HIM patients, the father and mother of case 1 and 2, their healthy brother and HIM related patients. IL-2–dependent T cell lines and EB virus transformed B cell lines were established and total RNA was extracted. Then entire cording region of CD40L was cloned and sequenced manually as described in Methods. In a female HIM and two male type I CVI (22) patients, no mutation was found.

and analyzed by PCR. Use of primers designed to copy the entire coding region of the PCR products of the CD40L gene transcripts showed normal length in all patients. The PCR-amplified cDNA of the CD40L coding region was then cloned into suitable vectors and fully sequenced in each case. Sequence analysis of cDNA obtained from brothers, case 1 and 2, revealed the same point mutation at nucleotide 475 (Fig. 1), turning the respective codon into a stop codon. This point mutation was reported to express a truncated CD40L on T cells (19). In a family study, the father and a healthy brother did not have the mutation, but the mother had an affected and an intact CD40 gene, which confirmed the X-linked trait. The cDNAs obtained from case 3 and 4 had new mutations. Case 3 had a point mutation at nucleotide 766, resulting in an exchange of valine for glutamic acid, and case 4 had a deletion at 692–693. In a female HIM and two male type I CVI (22) patients, no mutation was found. These results confirm a series of papers (17–20) that the patients diagnosed X-HIM have several mutations in CD40L gene.

Induction of immunoglobulin secretion in MNC from HIM patients. Recently, anti-CD40 plus IL-10 was reported to induce class switching from IgM to other isotypes of Ig in X-HIM patients (19, 21). MNC from X-HIM patients and a healthy brother of case 1 and 2 were stimulated with anti-CD40 (5C3)

Table I. IgM, IgG, and IgA Induction by Anti-CD40 in X-HIM

	Anti-CD40 + IL10	IgM (ng/ml)	IgG	IgA
Case 1	−	80	< 50	< 50
	+	1750	< 50	< 50
	+*	2160	< 50	< 50
Case 3	−	165	< 50	< 50
	+	3040	< 50	< 50
Case 4	−	150	< 50	< 50
	+	1840	< 50	< 50
Healthy*	−	110	260	220
Brother	+	1650	5140	6700

MNC (10^5/well) were stimulated with (+) or without (−) IL-10 plus anti-CD40 (5C3) for 14 d and IgM, IgG, and IgA secretion was examined by isotype specific ELISA. * Different anti-CD40 antibody (B-B20) was used. [‡] The healthy brother of case 1 (Fig. 1).

Table II. Induction of Proliferation in X-HIM B Cells by Cd4+ T Cell Clones

	B patient*	B brother[‡]
Medium	394±41 (c.p.m.)	511±32
Tc brother[‡]	4866±247	7134±915
Tc patient*	422±56	614±49
anti-CD40	2140±185	3327±416

B cells were stimulated with irradiated CD4+ T cell clones (Tc) or anti-CD40 (5C3) in anti-CD3 coated culture plates. Tc brother expresses CD40L but Tc patient expresses a truncated CD40L (19). Data represent mean [^{3}H]-TdR incorporation ±SD at day 5 of culture (c.p.m.). * Case 1 X-HIM patient. [‡] The healthy brother of case 1.

plus IL-10 for 14 d and Ig secretion was examined by Ig isotype specific ELISA. In the healthy brother's B cells, IgM, IgG, and IgA secretion was induced normally (Table 1). In HIM patients, however, IgG and IgA secretion was below detection although induction of IgM secretion was the same as that of healthy controls. When another lot of anti-CD40 (B-B20) was examined, production of IgG and IgA was also defective in case 1.

The stimulation of SAC plus anti-CD40 and IL-10 was reported to induce maximum Ig secretion in X-HIM (19). Patients' MNC were stimulated by these B-cell mitogens for 14 d. In a healthy control, induction of IgG and A secretion was observed significantly (Fig. 2). In all four X-HIM patients tested, however, there was no induction of IgG and IgA secretion. Again induction of IgM secretion was as much as healthy controls. These results suggest that anti-CD40 antibody does not rescue IgG or IgA production in X-HIM B cells.

Stimulation of X-HIM B cells with CD4+ T cell clone. It is uncertain whether anti-CD40 antibody provides optional signals

on interaction with CD40. In following experiments, B cells were stimulated with anti-CD3 activated CD4+ T cell clones instead of anti-CD40 antibody. It is reported that CD40L is preferentially expressed on activated CD4+ T cells (28). CD4+ T cell clones were prepared from case 1 and his healthy brother. B cells from case 1 and his healthy brother were stimulated with irradiated CD4+ T cell clones and examined for [^{3}H]TdR incorporation on day 5 of culture (Table II). CD4+ T cell clone from the healthy brother induced proliferation on both B cells significantly. However, CD4+ T cell clone from case 1 did not induce B cell proliferation at all. Anti-CD40, as a control, induced proliferation moderately on both B cells. These results suggest that both CD40L and this anti-CD40 can activate X-HIM B cells.

Induction of Ig secretion in X-HIM B cells by CD40L. Further experiments were carried out to examine whether CD40L on CD4+ T cell clones can correct the defective IgG and IgA production in X-HIM (Table III). Irradiated CD4+ T cell clones and IL-10 were cultured with X-HIM B cells. The CD4+ T cell clone from the healthy brother induced not only IgM but also IgG and IgA production by his B cells. However, the same T cell clone failed to induce IgG and IgA production by the HIM patient's B cells. Even in the healthy brother's B cells, CD4+ T cells from HIM patient did not induce Ig secretion at all.
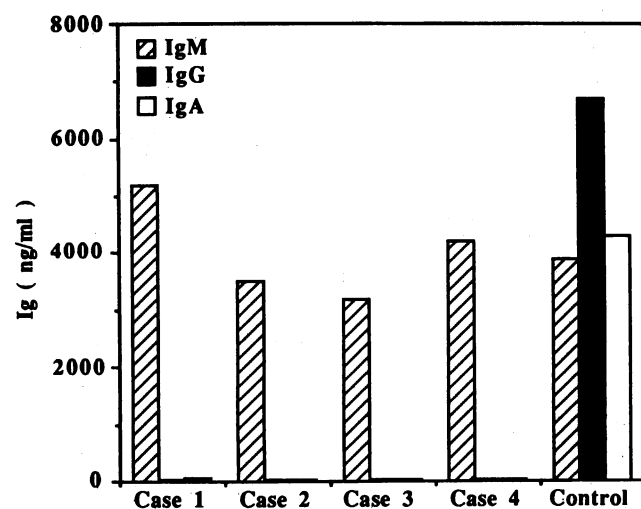

Figure 2. Induction of IgM, IgG, and IgA secretion by SAC in X-HIM. Peripheral blood MNC (10^5/well) were stimulated with SAC (0.001% vol/vol) plus anti-CD40 (5C3, 1 μg/ml) and IL-10 (100 ng/ml) for 14 d. IgM, IgG and IgA secretion was examined by isotype specific ELISA.

Table III. Induction of Ig Secretion by CD4+ T cell clones

	IgM	IgG	IgA
		ng/ml	
B bro. + med.	< 50	< 50	< 50
+T bro.*	2,160	3,340	1,840
+Tc pat.[‡]	< 50	< 50	< 50
B pat. + med.	< 50	< 50	< 50
+Tc bro.	1,420	< 50	< 50
+Tc pat.	< 50	< 50	< 50

B cells from X-HIM patient and his healthy brother were stimulated with irradiated CD4+ T cell clones (Tc) and IL-10 in anti-CD3 coated culture plates for 14 d. Ig secretion was examined by isotype specific ELISA. * CD4+ Tc from the healthy brother of case 1. [‡] CD4+ Tc from case 1 X-HIM patient.
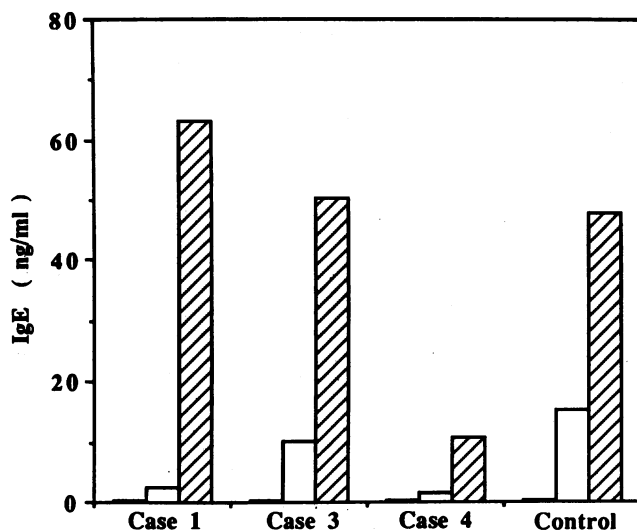
Figure 3. Induction of IgE secretion in X-HIM patients. Peripheral blood MNC (10^5/well) were stimulated with either anti-CD40 (5C3, 1 μg/ml, ■), anti-CD40 plus IL-4 (100 u/ml, □), or anti-CD40 plus IL-4 and IL-10 (100 ng/ml, ■), for 14 d. At the end of culture, supernatants were harvested and IgE secretion was examined by ELISA.

These results suggest that CD40L does not correct defective IgG or IgA production in X-HIM B cells.

Induction of IgE production in X-HIM patients. In further experiments, IgE production was examined. MNC from X-HIM patients were stimulated by anti-CD40, IL-4, and IL-10 for 14 d and IgE secretion was examined by ELISA. Anti-CD40 plus IL-4 induced small amount of IgE production (Fig. 3). When IL-10 was added to the culture, anti-CD40 plus IL-4 induced significant IgE production in every patient tested. The amount of IgE production in X-HIM patient is as much as that of the healthy control. In contrast, anti-CD40 alone did not induce IgE secretion at all. These result show that production of IgE can be induced by cross-linking of CD40.

Discussion

In all X-HIM families examined, several mutations including new mutations of CD40L were confirmed. Recent studies on X-HIM show that CD40L expression is incomplete and the defect is due to several mutations of genes coding for CD40L (17–20, 29). Our results support their finding that examination of the CD40L gene is critical for the diagnosis of X-HIM in contrast to other HIM and type I CVI whose B cells differentiate only IgM secreting cells (22).

The data of B cell function presented herein, however, are quite different from those of previous papers that describe signaling through CD40 rescues not only IgE but also IgG and IgA (18, 19, 21). In the present study, B cell defects of class switching for IgG and IgA were not rescued by any in vitro signaling through CD40 examined.

The reasons for the difference between former and present papers are not clear at present but several possibilities could be postulated. It is conceivable that the difference might be due to a different pattern of mutation in CD40L gene. However, in two patients (case 1 and 2), the same point mutation is found as in a patient T.G. (19). Even in a similar stimulation, such

as anti-CD40 plus SAC and IL-10 (19), the defective IgG and IgA production could not be rescued in case 1 and 2.

It is also conceivable that cross-linking of CD40 (5C3) did not provide sufficient signals for B cell activation. We examined another anti-CD40 (B-B20), but the antibody did not correct the defective IgG or IgA production. Instead of anti-CD40, B cells were stimulated with CD40L on CD4+ T cell clones. Activated CD4+ T cell clones, reported to express preferentially CD40L on their surface (28), induced IgG and IgA production in normal B cells but not in X-HIM B cells. Normal CD4+ T cells and anti-CD40, however, induced both proliferation and IgE secretion in X-HIM B cells, suggesting that the signals provided through CD40 are adequate for this receptor and further that IgG and IgA production is defective in X-HIM B cells in vitro.

As for IgE induction, our results are well in accordance with former papers (17–19, 21, 30). In the presence of IL-4, signaling through CD40 induces IgE secretion, suggesting that signaling through CD40 rescues IgE secretion in X-HIM. Hendriks et al. (31) show evidence that intrinsic Ig heavy chain class switch mechanism is intact in X-HIM. It is tempting to speculate that defective expression of CD40L would result in the inability to rearrange all Ig heavy chain genes downstream from the μ chain. In a healthy control, anti-CD40 plus IL-10 induces IgG and IgA but does not induce IgE production. IL-4, however, induced class switching for IgE preferentially, supporting the papers that IL-4 might be a in vitro switching factor specific for IgE and IgG_4 (27, 32).

It is also probable that in vitro signaling through CD40 may not provide adequate stimulation of class switching for IgG or IgA in peripheral blood X-HIM B cells. In another paper (33), we reported that sIgM+ peripheral blood B cells differentiate to IgM but not IgG or IgA secreting cells. In X-HIM, it is generally accepted that most of peripheral blood B cells bear IgM but not IgG or IgA on their surface (21, 30), suggesting that the defects of IgG and IgA production might be due to lack of sIgG and sIgA positive cells in peripheral blood. In case 1, 3, and 4, sIgG and sIgA positive cells were < 1% (data not shown). Indeed the reason why sIgG and sIgA positive cells are defective in X-HIM is not clear at present, but it is suggested that in vivo CD40-CD40L interaction might be necessary for IgG and IgA switching in X-HIM patients who lack of germinal center (1).

We hope that these observations will provide the basis for further and more sophisticated delineation of immunoregulatory abnormalities in this disease.

Acknowledgments

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