Comparison of Soluble and Membrane Bound HLA-Class I and DR Levels in Umbilical Cord Blood and Adult Peripheral Blood

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HLAs are membrane bound (membrane HLA = mHLA) or secreted as soluble forms (soluble HLA = sHLA) in plasma or serum. Umbilical cord blood (UCB) mHLA, from mononuclear cells (MNCs), and sHLA were quantified by flow cytometry and enzyme linked immunosorbant assay (ELISA), respectively, and compared to levels of MNC-mHLA and sHLA in adult peripheral blood (PB). The mean fluorescence intensity (MFI) of mHLA-I was 3-fold lower in UCB-MNCs than in adult PB-MNCs, however, due to higher cell numbers in UCB, total mHLA-I quantities per m*l* of blood were not different in UCB and adult PB. In addition, sHLA-I in UCB was significantly lower than in adult PB. The MFI of mHLA-DR from UCB-MNCs was significantly higher than the MFI of mHLA-DR in adult PB-MNCs, and the total mDR quantity (per ml of blood) in UCB was 3-fold higher than in adult PB. sHLA-DR concentrations, however, were equivalent in UCB and adult PB.
These findings indicate that fetal tissue or cells might excrete smaller quantities of both sHLA-I and sDR antigens than adult tissues or cells. Alternatively, fetal sHLA antigens might be unstable and degradated compared to those of adult.

Key words : Class I and DR, Human leukocyte antigen, Membrane form, Soluble form, Umbilical cord blood

INTRODUCTION

UCB has become an useful, alternative source for human stem cell transplantation for the treatment of various hematological disorders [1]. One advantage of UCB is its immunological immaturity [2, 3], which enables its use in most patients, even when HLA differ among the transplant pairs [4].

The expression of HLA molecules in UCB cells has been compared to that in adult PB [2, 3, 5-11]. The expression of HLA-class I is lower in UCB- than in adult PB- MNCs [5, 9]. The proportions of HLA-DR positive CD3+T cells, CD19+B cells or CD14+monocytes are lower in UCB than in adult PB [2, 6-8, 10]. These findings indicate that UCB-T or B-lymphocytes are resting, or non-activated, and also that monocyte antigen presentation is insufficient to activate host immune effectors.

sHLA-I have been quantified by ELISA in healthy individuals [13] and in individuals with various disorders such as cancer [14, 15] and autoimmune disease [16, 17] or in post-transplant patients [18, 19]. Conflicting comparisons of sHLA-I levels in UCB and adult PB have been reported [9, 10, 21]. sHLA-I antigens demonstrated immunosuppressive functions in *in vitro* conditions. Zavazava *et al.* [22], Popo *et al.* [23], and our group [24] revealed that sHLA-I induced apoptosis in CD8+ T cells, activated by allo-antigen, phytohemagglutinin, or viral (Epstein-Barr virus) antigen, respectively. sHLA-II concentrations, especially sDR, detected in human sera [25],

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were increased in autoimmune disorders [26] or following organ transplantation [27]. Specific tolerance to acetylcholine receptor epitopes was induced by soluble MHC-class II-peptide complexes in *in vitro* cloned T cells generated from myasthenia gravis patients [28]. However, until now, no report has been published about sHLA-DR in UCB sera.

The mHLA and sHLA levels from the same origin (samples) of UCB and adult PB were quantified by flow cytometric analysis or ELISA. The frequency of HLA expressing cells, as well as the fluorescence intensity per cell or volume of blood, were measured or calculated, especially for mHLA quantification. The total mHLA-I quantity per ml of blood was only marginally lower in UCB than in PB and the total mHLA-DR count was higher in UCB, though not statistically significant. sHLA-I levels were significantly lower in UCB than in PB. sHLA-DR levels did not differ in UCB and PB. The explanation and potential immunological significance of these results are discussed.

MATERIALS AND METHODS

Samples

A total of 16 PB samples were donated from normal healthy adults and 7 UCB samples were collected from full term deliveries in accordance with the Tokai University Committee on Clinical Investigation. The samples were stored at room temperature and processed within 24 hrs of collection.

Separation of MNCs and plasma

First, blood was centrifuged at 2500 rpm for 10 minutes. After the plasma was collected, the buffy coat was diluted with phosphate buffer saline (PBS) and loaded on Ficoll-Hypaque (1.077 g/cm^3) solution and centrifuged at 1500 rpm for 30 minutes to separate MNCs.

Flow cytometry

MNCs were incubated with unconjugated HLA-I MoAb (W6.32; from DAKO A/S, Glostrup, Denmark), then with FITC-conjugated goat anti-mouse immunoglobulins (Becton Dickinson, San Jose, CA). The cells were also double stained with PE-CD3, CD14, CD19 or CD20 monoclonal antibodies (MoAb) (Beckton Dickinson) together with FITC-HLA-DR MoAb (Beckton Dickinson). Finally, at least 1×10^4 viable cells were analyzed by FACS scan flow cytometry using Cellquest software (Beckton Dickinson).

sHLA-I ELISA

The system is described elsewhere [29], but briefly, anti-HLA-I MoAb, W6/32, (15 μg/ml) was plated on a 96 well microplate. After blocking with 0.05%Tween-20 PBS (TPBS) + 3 % bovine serum albumin, 1:50 diluted plasma samples were added. After washing, HRP-conjugated β2-microglobulin (Dako) (diluted 1:1000) was added to each well. Finally, the reaction was completed with the TMB-peroxidase EIA Substrate Kit (BioRad Labs, Herculus, CA) and optical density (O.D.) was measured by a THERMOmax microplatereader (Molecular Device, Nenlo Park, CA). The O.D. was converted to ng/ml using a standard curve constructed with standard antigens, which were prepared from the culture supernatant of C1R neo B7 (donated from Dr. F. Carl Grumet, Stanford University).

sHLA-DR ELISA

In brief, 96 well-plates were coated with anti-HLA-DR MoAb, L243 (Beckton Dickinson) at a concentration of 15 μg/ml. The samples at 1:2 of dilution were added, after blocking with 0.05 % TPBS + 3 % bovine serum albumin. After washing with TPBS, each well was incubated with anti-HLA-DR MoAb, CR3/43 (Boehringer Mannheim GmbH, Germany). After washing, peroxidase-conjugated anti-mouse IgG1 (Zymed) (diluted 1:1000) was added to each well, followed by the same procedure as for sHLA-I ELISA. The O.D. was changed to ng/ml using a standard curve constructed with purified HLA-class II antigens, which were donated by Dr. H. Grosse-Wilde (Essen University, Essen, Germany). Details of the protocol are described in a previous manuscript [30].

Statistical analysis

Results were expressed as mean ± standard error (SE). The difference in mean values between the two groups was analyzed by the Mann-Whitney U test.

RESULTS

Total mHLA-I and DR quantities in UCB and adult PB

As shown in Fig.1-a, over 80 % of UCB cells expressed HLA-I and a statistical significant difference of MFI was not detected between UCB and adult PB ($p = 0.08$). Representative histograms of HLA-I expression are shown in Fig. 1-b. The total number of MNCs per ml of blood was consistently higher (approximately twice as high) in UCB than in adult PB. We calculated the total mHLA-I quantity per ml of blood by multiplying the MFI by the frequency of HLA-I positive cells and by the total MNC number per ml, divided by 10^6. As shown, the total mHLA-I concentrations were equivalent in UCB and adult PB.

The frequencies of HLA-DR expression was similar in UCB and adult PB (Fig. 2-a), however, the MFI was unexpectedly higher in UCB than in adult PB. When each cell fraction was analyzed, the frequencies of CD14+/DR+, CD19+/DR+ or 20+/DR+ cells were not different between UCB and adult PB (Fig. 2-b). Meanwhile, the MFI of DR expressing cells was significantly higher in UCB-CD3+ ($p = 0.02$), -CD19+ ($p = 0.007$), and -CD20+ cells ($p = 0.02$) compared to the adult-PB counterparts. Representative dot grams are shown in Fig. 2-c. As with mHLA-I, the total mHLA-DR per ml of blood was calculated by multiplying MFI by the frequency of HLA-DR positive cells and by the total MNC numbers per ml, divided by 10^6. mHLA-DR concentrations per ml of blood were higher in UCB (Fig.

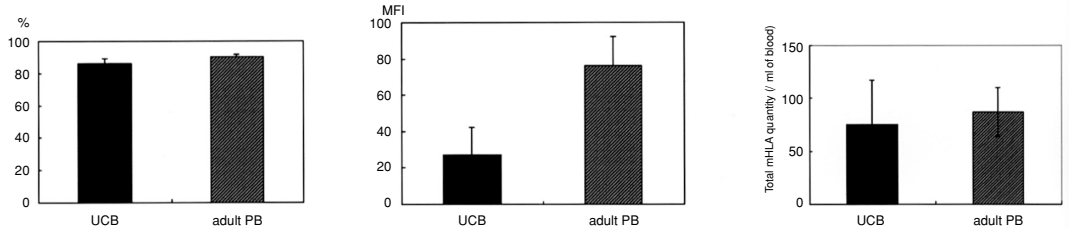

Fig. 1-a HLA-I expression in UCB- and adult PB-MNCs. Frequency (left) of HLA-I expressing cells and MFI (middle) of HLA-I expression were analyzed by flow cytometry. Total mHLA-I quantitity per ml of blood cells was calculated by multiplying MFI by the frequency of HLA-I positive cells and by total MNC numbers per ml divided by 10^6 (right).

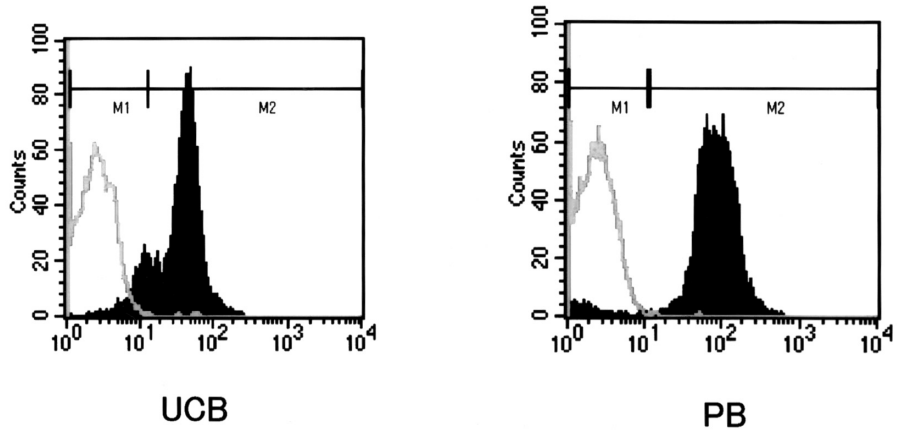

Fig. 1-b Histograms of HLA-I expression in UCB- (left) and adult−PB- (right) MNCs. Data is derived from 7 UCB- and 16 adult PB-samples. The open histogram represents cells stained with isotype-matched control immunoglobulin.

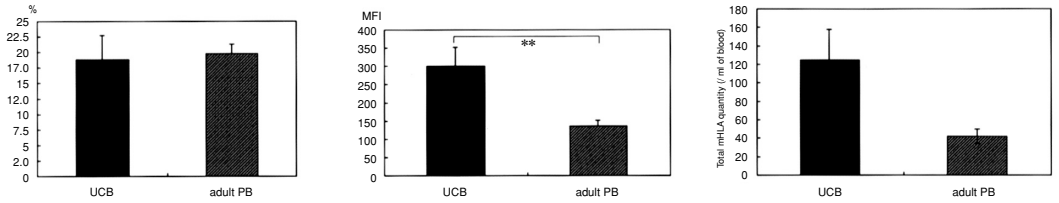

Fig. 2-a HLA-DR expression in UCB- and adult PB-MNCs. Frequency (left) of HLA-DR expressing cells and MFI (middle) of HLA-DR expression were analyzed by flow cytometry. Total mHLA-DR quantitity per ml of blood cells was calculated by multiplying MFI by the frequency of HLA-DR positive cells and by total MNC numbers per ml divided by 10^6 (right).

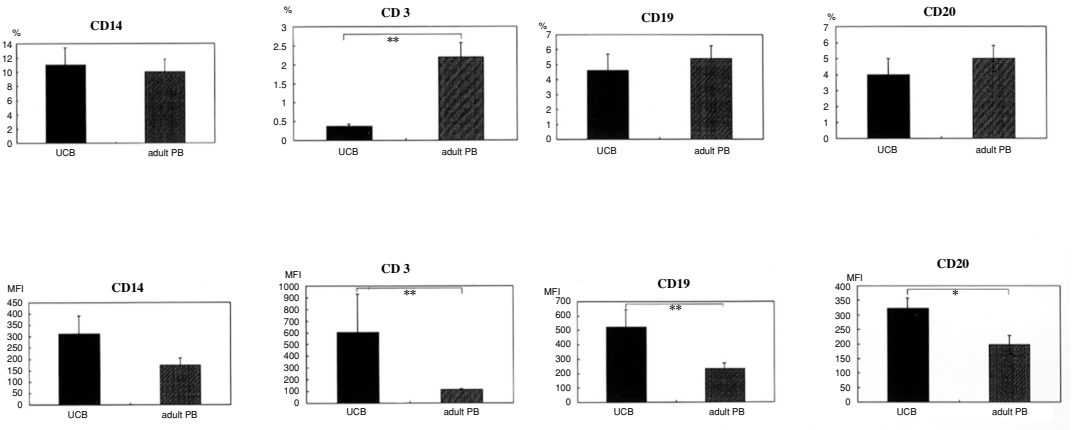

Fig. 2-b Frequencies (upper) of HLA-DR positive CD14+, CD3, CD19+ or CD20+ cells and MFI (lower) of DR antigens in each fraction. Statistical significance between UCB and adult PB is shown by asterisks (*; $p < 0.05$, **; $p < 0.01$).

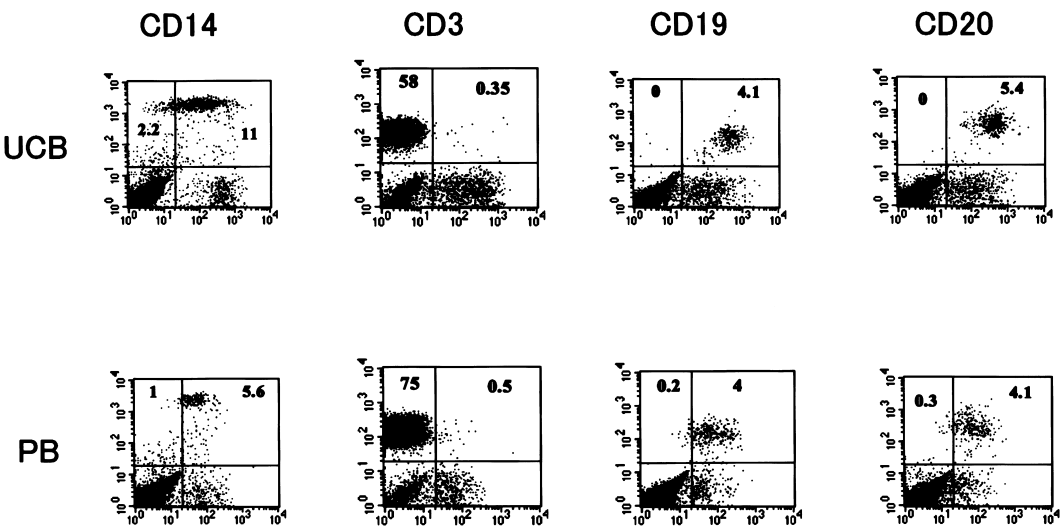

Fig. 2-c Dot plots showing the HLA-DR expression in UCB- or adult PB-CD14+, CD3+, CD19+ or CD20+ cells. Data are derived from 7 UCB and 16 adult PB samples.

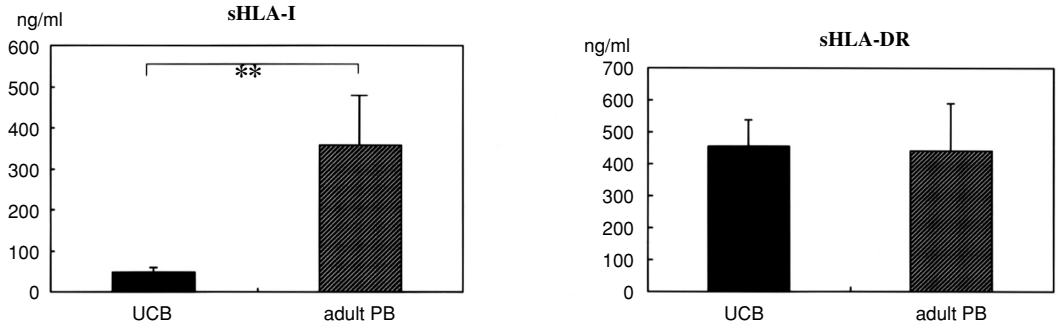

Fig. 3 Soluble HLA-I (left) and DR (right) levels in UCB and adult PB sera. Statistical significance between UCB and adult PB was shown as for sHLA-I ($p < 0.01$).

2-a), although statistical significance was not obtained ($p = 0.06$).

sHLA-I and DR concentrations in UCB and adult PB

sHLA-I was significantly lower ($p = 0.003 < 0.01$) in UCB than in adult PB plasma (Fig. 3). In contrast, sHLA-DR concentrations were similar in UCB and adult PB.

DISCUSSION

The present study revealed that cell-surface membrane bound HLA-I expression in UCB-MNCs was approximately 3-fold lower than in adult PB-MNCs, even if the difference did not reach a level of statistical significance. sHLA-I levels were also significantly lower in UCB than in adult PB. Puri [9] and Inostroza *et al.* [21] also reported that sHLA concentrations were lower in UCB than in adult PB sera. Puri reported that reduced HLA-I expression by neonatal leukocytes was responsible for the lower sHLA-I levels [9]. sHLA-I can exist as three different sized molecules: a 36 KDa breakdown molecule, a 39 KDa spliced molecule, and a 42 KDa shedded form. Puris *et al.* [9] also reported that the distribution of sHLA-I in UCB sera did not differ from that in adult sera, indicating no preferential reduction of any particular form of sHLA-I in UCB sera. Our findings are in agreement with their report, and we propose that membrane and surface HLA-I antigens are controlled by a common mechanism of regulation or signaling of production.

UCB contained approximately 2-fold higher numbers of total cells or MNCs per ml of blood than adult PB (data not shown). Therefore, even if UCB cells secrete lower amounts of sHLA-I, the total accumulated quantity of sHLA-I would be expected to be elevated close to adult PB levels. When "a total mHLA-I quantity per ml of blood" was calculated, only a slight difference was observed between UCB and adult PB. HLA-I are expressed in almost all nucleated cells, and the circulating sHLAs are derived from organs or tissues such as the liver, an especially active producer as well as from MNCs [18, 31]. Recently, Demaria *et al.* reported the release of β2-microglobulin (β2 m)-free-HLA-class I heavy chain (HC) from cell surfaces by membrane metalloproteinases, and their subsequent re-association with β2 m [32]. They also reported that exogenous β2 m binds only to peptide-confirmed HC antigens, and is protected from cleavage, in the same way as the MPase inhibitor. Endogeneous cancer-derived peptides have also been recovered from sHLA-I in patients' sera [33]. Taken together, two possible explanations for these observations include: 1) MPase activity on UCB cell membranes is lower than in adult PB cell membranes or, 2) peptide-free non-confirmed HC concentrations are higher in UCB than in adult PB. Alternatively, peptide-unbound HLAs could be unstable and degraded, as shown by Chersi *et al.* [34].

Several studies have shown that HLA-class II, DR antigens are expressed weakly or in a lower proportion in UCB-T/B lymphocytes or -monocytes than in adult PB-T/B lymphocytes or -monocytes [2, 6-8, 10]. Higher DR expression on CD20+B cells or an equivalent level of expression on monocytes have been proposed as explana-

tions by Keever or Harris *et al.* [5, 6] Our results show that the frequency of DR positive monocytes or B cells (not T cells) is the same in UCB and adult PB. An especially interesting finding is that the intensity of DR antigens on UCB-monocytes and -B cells was 2-fold higher than in adult PB-monocytes and -B cells. Such relatively high HLA-DR expression, especially manifested by antigen-presenting cells (APCs), could trigger a strong allo-reaction in to DR-mismatched recipients. Furthermore, CD3+T cells expressed a significantly higher DR level, even though the UCB-T cell frequencies were lower than in adult−PB-T cells. A potential explanation is that a small proportion of UCB-T lymphocytes may have been activated by particular gestational antigens. The function of UCB-T cells should be investigated, especially with regard to allo-immune reactions.

We examined, for the first time, the content of sHLA-DR in UCB−sera; sHLA-DR concentrations were virtually equivalent in UCB and adult PB sera, even though the total mDR concentration in UCB was 3 times higher than that in adult PB. Fetal tissue or cells might have a reduced ability to secrete sHLA-DR. It is still unknown which organs or tissues contribute to the production of sHLA-DR in human sera. However, APCs, such as monocytes, B cells or dendritic cells, which not only circulate in blood but also reside in peripheral tissues, express mHLA-DR to present foreign antigens to CD4+T cells. Therefore, sDR antigens may derive in considerable amounts from peripheral tissues through encounters with foreign antigens after birth. Garban *et al.* [11] reported that HLA-DR molecules on UCB-B cells were devoid of stably bound peptides. Such HLA-DR, if separated from cell surface membranes, might be fragile and easily degraded in UCB sera.

sHLA is considered unique because it shows immunomodulatory and suppressive functions *in vitro* [22-24, 35]. Human allograft acceptance is closely correlated with sHLA antigen-driven immune regulation [17]. Burlingham [36] mentioned that maternal sHLA proteins might induce tolerance via indirect presentation of maternal allopeptides to fetal T cells. The effect of non-inherited maternal antigens (NIMA), in allo-organ or stem cell engraftment, might be explained by sHLA [37]. sHLA can be easily allo-typed by ELISA [27, 38]. Maternal-derived sHLA-I or −DR in UCB−sera, and its contribution to the depressed immune reaction to NIMA, should be investigated in the future.

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REFERENCES

1) Rubinstein P, Carrier C, Scaradavou A: Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med, 339: 1565-1577, 1998.
2) Harris DT, Schumacher MJ, Locascio J: Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. Proc Natl Acad Sci U S A, 89: 10006-10010, 1992.
3) Roncarolo MG, Bigler M, Ciuti E, Martino S, Tovo PA: Immune responses by cord blood cells. Blood Cells, 20: 573-585, 1994.
4) Wagner JE, Rosenthal J, Sweetman R: Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. Blood, 88: 795-802, 1996.
5) Keever CA, Abu-Hajir M, Graf W: Characterization of the alloreactivity and anti-leukemia reactivity of cord blood mononuclear cells. Bone Marrow Transplant, 15: 407-419, 1995.
6) Harris DT, Schumacher MJ, LoCascio J, Booth A, Bard J, Boyse EA: Immunoreactivity of umbilical cord blood and post-partum maternal peripheral blood with regard to HLA-haploidentical transplantation. Bone Marrow Transplant, 14: 63-81, 1994.
7) Milosevits J, Pocsik E, Schmidt B: Immunophenotypic and functional characteristics of haemopoietic cells from human cord blood. Scand J Immunol, 42: 493-500, 1995 .
8) Kampalath B, Cleveland RP, Kass L: Reduced CD4 and HLA-DR expression in neonatal monocytes. Clin Immunol Immunopathol, 87: 93-100, 1998.
9) Puri S, Shieh DC, Canavan A, Kao KJ: Quantitation and characterization of plasma HLA in neonates of different gestational ages. Tissue Antigens, 42: 67-71, 1993.
10) Stiehm ER, Sztein MB, Steeg PS: Deficient DR antigen expression on human cord blood monocytes: reversal with lymphokines. Clin Immunol Immunopathol, 30: 430-436, 1984.
11) Garban F, Ericson M, Roucard C: Detection of empty

HLA class II molecules on cord blood B cells. Blood, 87: 3970-3976, 1996.
12) van Rood JJ, van Leeuwen A, van Santen MC: Anti HL-A2 inhibitor in normal human serum. Nature, 226: 366, 1970.
13) Hagihara M, Shimura T, Yamamoto K, Takebe K, Munkhbat B, Tsuji K: Soluble HLA class I and class II in Japanese. Hum Immunol, 40: 171-173, 1994.
14) Shimura T, Hagihara M, Yamamoto K: Quantification of serum-soluble HLA class I antigens in patients with gastric cancer. Hum Immunol, 40: 183-186, 1994.
15) Shimura T, Tsutsumi S, Hosouchi Y: Clinical significance of soluble form of HLA class I molecule in Japanese patients with pancreatic cancer. Hum Immunol, 62: 615-619, 2001.
16) Hagihara M, Shimura T, Yamamoto K: Clinical significance of serum soluble HLA class I antigens in systemic lupus erythematosus. Tokai J Exp Clin Med, 18: 61-64, 1993.
17) VanBuskirk AM, Burlingham WJ, Jankowska-Gan E: Human allograft acceptance is associated with immune regulation. J Clin Invest, 106: 145-55, 2000.
18) Hagihara M, Munkhbat B, Kanai N: Serum soluble human leucocyte antigen class I in paediatric liver transplantation with live, related donors. Transpl Immunol, 5: 219-224, 1997.
19) Puppo F, Brenci S, Ghio M: Serum HLA class I antigen levels in allogeneic bone marrow transplantation: a possible marker of acute GVHD. Bone Marrow Transplant, 17: 753-758, 1996.
20) Webb BJ, Bochan MR, Montel: The lack of NK cytotoxicity associated with fresh HUCB may be due to the presence of soluble HLA in the serum. Cell Immunol, 159: 246-261, 1994.
21) Inostroza J, Ferrada J, Navarrete C, Sorensen RU: Soluble histocompatibility class I antigens and beta 2-microglobulin in pregnant females and cord blood samples. Hum Immunol, 54: 63-68, 1997.
22) Zavazava N, Kronke M: Soluble HLA class I molecules induce apoptosis in alloreactive cytotoxic T lymphocytes. Nat Med, 2: 1005-1010, 1996.
23) Puppo F, Contini P, Ghio M: Soluble human MHC class I molecules induce soluble Fas ligand secretion and trigger apoptosis in activated CD8(+) Fas (CD95)(+) T lymphocytes. Int Immunol, 2: 195-203, 2000.
24) Gansuvd B, Hagihara M, Munkhbat B: Inhibition of Epstein-Barr virus (EBV)-specific CD8+ cytotoxic T lymphocyte (CTL) activity by soluble HLA class I *in vitro*. Clin Exp Immunol, 119: 107-114, 2000.
25) Jendro M, Goronzy JJ, Weyand CM: Structural and functional characterization of HLA-DR molecules circulating in the serum. Autoimmunity, 8: 289-296, 1991.
26) Weyand CM, Jendro M, Goronzy JJ: Soluble HLA-DR molecules in patients with HLA class II versus class I associated disorders. Autoimmunity, 8: 281-287, 1991.
27) Filaci G, Contini P, Brenci S: Increased serum concentration of soluble HLA-DR antigens in HIV infection and following transplantation. Tissue Antigens, 46: 117-123, 1995.
28) Nicolle MW, Nag B, Sharma SD: Specific tolerance to an acetylcholine receptor epitope induced *in vitro* in myasthenia gravis CD4+ lymphocytes by soluble major histocompatibility complex class II-peptide complexes. J Clin Invest, 93: 1361-1369, 1994.
29) Hagihara M, Shimura T, Takebe K: Serum concentrations of soluble HLA-class I and CD8 forms in patients with viral hepatic disorders. J Gastroenterol, 132: 338-343, 1997.
30) Hagihara M, Hosoi K, Kagawa T: Serum soluble HLA-DR antigens in autoimmune hepatitis. Autoimmunity, 31: 85-93, 1999.
31) Mathew JM, Shenoy S, Phelan D, Lowell J, Howard T, Mohanakumar T: Biochemical and immunological evaluation of donor-specific soluble HLA in the circulation of liver transplant recipients. Transplantation, 62: 217-223, 1996.
32) Demaria S, Bushkin Y: Soluble HLA proteins with bound peptides are released from the cell surface by the membrane metalloproteinase. Hum Immunol, 61: 1332-1338, 2000.
33) Barnea E, Beer I, Patoka R: Analysis of endogenous peptides bound by soluble MHC class I molecules: a novel approach for identifying tumor-specific antigens. Eur J Immunol, 32: 213-222, 2002.
34) Chersi A, Garzillo C, Butler RH, Tanigaki N: Allele- and temperature-dependency of *in vitro* HLA class I assembly. Hum Immunol, 62: 858-868, 2001.
35) Claus R, Werner H, Schulze HA, Walzel H, Friemel H: Are soluble monocyte-derived HLA class II molecules candidates for immunosuppressive activity? Immunol Lett, 26: 203-210, 1990.
36) Burlingham WJ: Soluble MHC, immunoregulation, and tolerance: a progress report. Hum Immunol, 61: 1316-1319, 2000.
37) McDonald JC, Adamashvili I, Zibari GB: Serologic allogeneic chimerism. Transplantation 64: 865-871, 1997.
38) Borelli SD, Ferreira E, Oliveira AM, Krishnaswamy S, Hiraki DD, Grumet FC: Specific sHLA in healthy donors and donor-specific sHLA in renal transplant patients. Hum Immunol, 60: 430-4, 1999.