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Regulatory CD8$^+$ T cells induced by exposure to all-trans retinoic acid and TGF-β suppress autoimmune diabetes

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ABSTRACT

Antigen-specific regulatory CD4$^+$ T cells have been described but there are few reports on regulatory CD8$^+$ T cells. We generated islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific regulatory CD8$^+$ T cells from 8.3-NOD transgenic mice. CD8$^+$ T cells from 8.3-NOD splenocytes were cultured with IGRP, splenic dendritic cells (SpDCs), TGF-β, and all-trans retinoic acid (ATRA) for 5 days. CD8$^+$ T cells cultured with either IGRP alone or IGRP and SpDCs in the absence of TGF-β and ATRA had low Foxp3$^+$ expression (1.7 ± 0.9% and 3.2 ± 4.5%, respectively). In contrast, CD8$^+$ T cells induced by exposure to IGRP, SpDCs, TGF-β, and ATRA showed the highest expression of Foxp3$^+$ in IGRP-reactive CD8$^+$ T cells (36.1 ± 10.6%), which was approximately 40-fold increase compared with that before induction culture. CD25 expression on CD8$^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA was only 7.42%, whereas CD103 expression was greater than 90%. These CD8$^+$ T cells suppressed the proliferation of diabetogenic CD8$^+$ T cells from 8.3-NOD splenocytes *in vitro* and completely prevented diabetes onset in NOD-scid mice in cotransfer experiments with diabetogenic splenocytes from NOD mice *in vivo*. Here we show that exposure to ATRA and TGF-β induces CD8$^+$Foxp3$^+$ T cells *ex vivo*, which suppress diabetogenic T cells *in vitro* and *in vivo*.

1. Introduction

The adaptive immune response is initiated by T cells that express receptors for diverse antigens. The repertoire of T cell receptors is regulated by positive selection and negative selection. Autoreactive T cells are usually deleted by negative selection in the thymus [1]. However this process is not exhaustive: T cells expressing receptors with low affinities for self-antigens are usually present in peripheral organs and may cause autoimmune disease if activated. Suppressor mechanisms for self-reactive T cells in peripheral organs have been presumed. The concept of T cell suppression of the immune response was first proposed by Gershon and Kondo for regulatory CD8$^+$ T cells [2]. There are few studies on regulatory CD8$^+$ T cells because of the difficulty of identifying regulatory CD8$^+$ T cell populations and their mechanisms of action. The CD4$^+$ population of regulatory T cells (Tregs) was first described by Sakaguchi et al. as CD4$^+$CD25$^+$ T cells [3]. Although CD25 expression has been used as a marker to identify Tregs, it is not specific for Tregs because it is expressed by all activated T cells. Recent studies revealed that the forkhead/winged helix family (Foxp3) transcription factor is a master switch specific to CD4$^+$CD25$^+$ Tregs that induces differentiation of naive T cells into the Treg lineage and maintains their suppressive function [4,5].

The CD4$^+$CD25$^+$ T cells described by Sakaguchi et al. originated in the thymus and developed their suppressive function in peripheral organs. On the other hand, Luo et al. revealed that transforming growth factor-β (TGF-β) and β-cell peptide-pulsed dendritic cells from NOD mice induced CD4$^+$CD25$^+$Foxp3$^+$ T cells from naïve T cells in BDC2.5-NOD splenocytes, which suppressed diabetes onset in cotransfer experiments [6]. In contrast to the regulatory cells in the thymus, BDC2.5 CD4$^+$CD25$^-$ T cells can be switched to CD4$^+$CD25$^+$Foxp3$^+$ T cells by regulatory cytokines such as TGF-β.

Vitamin A (retinol) and its metabolites (retinoids) are a group of potent natural or synthetic molecules which act as modulators for a variety of inflammatory and immunological events in immune system. The mechanism of this molecule has been reported as suppression of inflammatory immune cells, modulation of the function of immune cells and production of several cytokines [7]. All-trans retinoic acid (ATRA), a potent retinoids, has been clinically used to treat acute leukaemia and acne vulgaris [8,9]. Now it has also been reported as having a potential of generating CD4$^+$ Tregs [10,11]. In addition, dendritic cells purified from the small intestine were found to undergo a high level of CD4$^+$ Treg conversion when exposed to TGF-β and ATRA that was highly expressed in GALT [12].

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Recent publications have shown that the regulatory T cell family consists not only of $CD4^+$ T cells but also $CD8^+$ T cells [13–17], presuming that regulatory $CD8^+$ T cells can be induced from these naïve $CD8^+$ T cells in 8.3-NOD mice in an adequate milieu. In this study, we generated regulatory $CD8^+$ T cells from transgenic 8.3-NOD mice expressing CD8-TCR specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in β-cells [18]. $CD8^+$ T cells exposed to ATRA and TGF-β exhibited regulatory functions *in vitro* and *in vivo*.

2. Materials and methods

2.1. Mice

NOD/Shi/Kbe mice were maintained at the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. The cumulative incidence of diabetes is 85% in females and 30% in males at 40 weeks of age. NOD-scid mice were purchased from Clea Japan (Osaka, Japan). 8.3-NOD mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were treated according to the Guidelines for Animal Experimentation of Kobe University School of Medicine.

2.2. Antibodies and reagents

FITC-conjugated anti-mouse monoclonal antibodies, CD8 (53–6.7) and CD103 (M290), were purchased from Pharmingen (San Diego, CA), as were the phycoerythrin (PE)-conjugated anti-mouse monoclonal antibodies, CD4 (L3T4), CD8 (53–6.7), and B220 (RA3–6B2), peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD4 monoclonal antibody (L3T4), and the APC-conjugated anti-mouse CD25 monoclonal antibody (3C7). The PE-conjugated anti-mouse Foxp3 monoclonal antibody (FJK-16s) was purchased from eBioscience (San Diego, CA). Anti-CD11c (N418) microbeads and the CD8 isolation kit were purchased from Miltenyi Biotec (Bergisch–Gladbach, Germany). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Dojindo (Kumamoto, Japan).

2.3. Cell purification and culture

Splenic dendritic cells (SpDCs) were purified from 8- to 12-week-old 8.3-NOD mice splenocytes using CD11c-magnetic beads. After selection using an autoMACS magnetic cell sorter (Miltenyi Biotec), $CD8^+$ T cells were purified by depletion of CD4-, B220-, CD49b-, CD11b-, and Ter-119-positive cells. To study the proliferation of $CD8^+$ T cells, some cells were stained with 10 μM CFSE for 15 min at 37 °C followed by two washes with HBSS at 4 °C. All cells were cultured in an atmosphere containing 5% CO_2 at 37 °C in 96-well tissue culture plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM sodium pyruvate, 10 mM Hepes buffer, 50 IU/ml penicillin, 50 μg/ml streptomycin, 40 μg/ml gentamycin and 5×10^{-5} M 2-mercaptoethanol. $CD8^+$ T cells (6.0×10^4) were cultured with 0.1 μM of IGRP alone or with IGRP and 2.0×10^4 of SpDCs as controls. In addition to IGRP and SpDCs, $CD8^+$ T cells in other groups were cultured with 2 ng/ml of TGF-β or with TGF-β and 10 nM of ATRA to induce the regulatory cells. Five-days later, cells were harvested and stained with several antibodies and propidium iodide (PI); only PI-negative cells were analyzed using the FACS 440 flow cytometer (Becton Dickinson, San Jose, CA). For each group, the $CD8^+$ T cells were cultured as described previously and harvested; only $CD8^+$ T cells were selected using the magnetic beads. $CD8^+$ T cells cultured with IGRP alone were designated I cells, those cultured with IGRP and SpDCs were designated ID cells, those cultured with IGRP, SpDCs, and TGF-β were designated IDT cells, and those cultured with IGRP, SpDCs, TGF-β, and ATRA were designated IDTA cells.

2.4. Proliferation assays

$CD8^+$ T cells from 8.3-NOD splenocytes that were selected using magnetic beads were stained with 10 μM CFSE for 15 min at 37 °C and were washed twice. Induced $CD8^+$ T cells (1.0×10^5) (I cells, ID cells, IDT cells, or IDTA cells) and CFSE-stained fresh $CD8^+$ T cells (1.0×10^5) were cultured together with 0.1 μM of IGRP. After 3 days, all cells were harvested and only $CFSE^+$ cells were analyzed using flow cytometry. To further examine the suppressor activity of induced $CD8^+$ T cells *in vitro*, freshly-isolated effector $CD8^+$ T cells (1.0×10^5) (E) were cultured with induced $CD8^+$ T cells (IDT cells or IDTA cells) (R) at the indicated ratios in the presence of 0.1 μM of IGRP.

2.5. Adoptive transfer

$CD8^+$ T cells (6.0×10^6) from 8.3-NOD splenocytes cultured with IGRP alone or IGRP, SpDCs, TGF-β, and ATRA were intravenously cotransferred into 8-week-old NOD-scid mice with 1×10^7 diabetogenic NOD splenocytes. The recipients were monitored for diabetes onset by testing urine glucose level twice weekly. Diabetes was defined as a blood glucose concentration greater than 250 mg/dl (13.9 mmol/l) on two consecutive days.

2.6. Statistical analysis

Statistical analysis of the incidence of diabetes was performed using the log-rank test. Statistical analyses of flow cytometric data were performed using the Mann–Whitney *U* test. A p value less than 0.05 was considered significant. All data are presented as the means ± SD.

3. Results

3.1. Induction of $CD8^+Foxp3^+$ T cells from splenocytes in 8.3-NOD mice

The prevalence of $CD8^+Foxp3^+$ T cells among splenocytes in 7-week-old 8.3-NOD mice was first analyzed using flow cytometry to evaluate "natural" $CD8^+Foxp3^+$ T cells. The population of $CD8^+$ T cells in the spleen from 8.3-NOD mice exceeded 50% of splenocytes, which was higher than that of littermate NOD mice (Fig. 1A). The population of $CD8^+Foxp3^+$ T cells in the spleen from 8.3-NOD mice was no more than 1.0% of splenocytes, which was equal to that in littermate NOD mice (Fig. 1B).

SpDCs and $CD8^+$ T cells were cultured for inducing regulatory $CD8^+$ T cells *ex vivo*. SpDCs were selected from the splenocytes of 8.3-NOD mice using CD11c-magnetic beads. $CD8^+$ T cells were negatively selected using magnetic beads followed by labeling with CFSE. CFSE-labeled $CD8^+$ T cells (6.0×10^4) were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells) or with IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Fig. 1C and D show the *in vitro* proliferation of $Foxp3^+$ cells in cultured $CD8^+$ T cells from 8.3-NOD splenocytes. Only 1.7 ± 0.9% of I cells and 3.2 ± 4.5% of ID cells expressed Foxp3, whereas 8.6 ± 6.7% of IDT cells and 21.4 ± 4.2% of IDTA cells expressed Foxp3. IDTA cells showed the highest expression of Foxp3 and significantly higher expression than I cells ($^*p = 0.028$). To further examine whether the efficiency of conversion to Tregs can be increased, we also cultured $CD8^+$ T cells from 8.3-NOD mice with titrating concentrations of ATRA (0, 1, 5, 10, and 20 nM) in the presence of constant IGRP, splenic DC, and TGF-β concentrations. The percentage of $Foxp3^+$

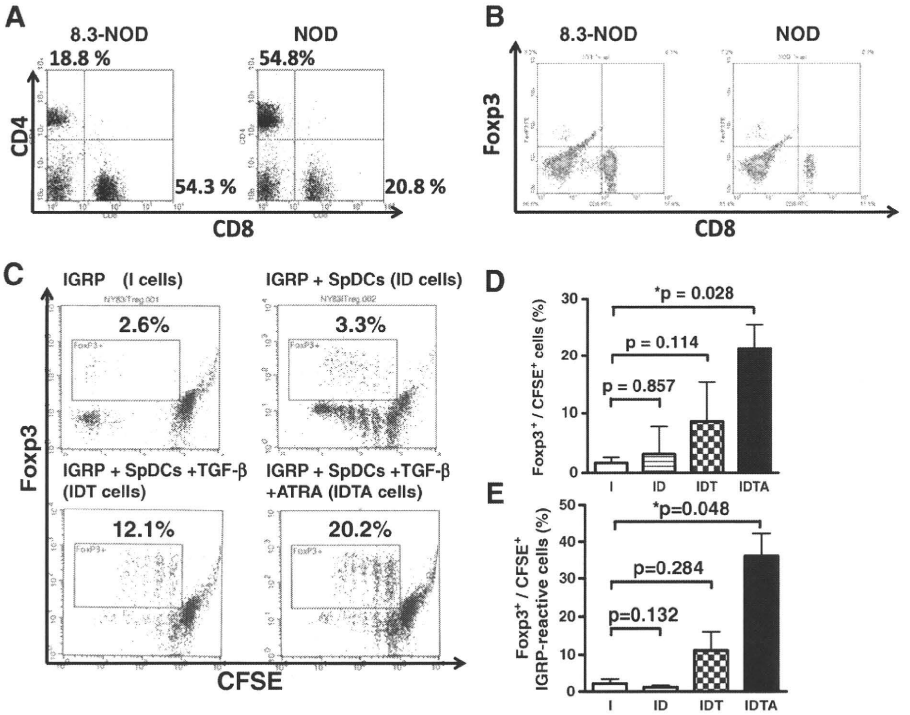

Fig. 1. (A, B) Splenocytes from 7-week-old 8.3-NOD or NOD mice were stained with anti-CD4 and CD8 antibodies (A) or anti-FoxP3 and CD8 antibodies (B). A representative example of four separate experiments is shown. (C, D, E) CFSE-labeled $CD8^+$ T cells (6.0×10^4) selected from the splenocytes of 7-week-old 8.3-NOD mice were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells), or IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Five days later, all cells were harvested and gated on PI-negative and CFSE positive cells. A representative example (C), percentage of $Foxp3^+$ cells in $CFSE^+$ cells ($^*p = 0.028$, I vs. IDTA) (D) and percentage of $Foxp3^+$ cells in IGRP-reactive $CFSE^+$ cells ($^*p = 0.048$, I vs. IDTA) (E) are shown.

cells in 10 nM of ATRA was the highest among these titrating concentrations (data not shown). Among IGRP-responded $CD8^+$ T cells, 36.1 ± 10.6% of IDTA cells showed Foxp3 expression, which was approximately 40-fold increase compared with that before induction culture and significantly higher than that of I cells ($^*p = 0.048$) (Fig. 1E).

3.2. Cell surface marker of $CD8^+Foxp3^+$ T cells

To determine which surface markers were expressed on $CD8^+Foxp3^+$ T cells, IDT cells or IDTA cells were stained with CD25 and CD103 antibodies (Fig. 2). In contrast to $CD4^+Foxp3^+$ T cells, only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive.

3.3. In vitro suppression assay

To examine the suppressor activity of $CD8^+$ T cells *in vitro*, 1.0×10^5 of the CFSE-labeled effector $CD8^+$ T cells were cultured with IGRP and 1.0×10^5 of I, ID, IDT, or IDTA cells (Fig. 3A). Proliferation of $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was compared with that induced by IGRP alone. % Suppression of each proliferation was −1.8 ± 7.6%, 2.9 ± 9.0%, 1.0 ± 8.5%, and 12.9 ± 8.9%, respectively, and the presence of I, ID, or IDT cells did not markedly suppress proliferation of IGRP-reactive $CD8^+$ T cells. In addition, there was no significant suppression in the presence of ID cells or IDT cells compared with the presence of I cells ($p > 0.05$). However, only the presence of IDTA cells significantly suppressed proliferation of IGRP-reactive effector $CD8^+$ T cells than the presence of I cells ($^*p = 0.009$) (Fig. 3B). Furthermore, the suppressive activity seems to be strengthened in a E:R ratio-dependent manner, though not all of IDTA cells express Foxp3 (Fig. 3C).

3.4. In vivo suppression assay

Because IDTA cells alone have suppressor activity for diabetogenic antigen-specific $CD8^+$ T cells *in vitro*, *in vivo* suppressor activity was examined using a diabetes-transfer model. IDTA cells or I cells were intravenously transferred into NOD-scid mice with diabetogenic splenocytes from NOD mice. All of five mice injected with diabetogenic NOD splenocytes alone and six mice injected with diabetogenic splenocytes and I cells became diabetic by 44 days after transfer. In contrast, none of the five mice injected with diabetogenic splenocytes and IDTA cells became diabetic in this cotransfer experiment ($^*p = 0.01$, IDTA vs. control) (Fig. 4). In another set of experiment, none of mice injected with diabetogenic splenocytes and IDTA cells became diabetic even at 150 days after transfer which was more than 30 days after last positive control became diabetic (data not shown). These findings suggest that $CD8^+$ T cells induced by exposure to SpDCs, TGF-β, and ATRA have suppressor activity against the autoimmune response *in vivo* and that the disease should be suppressed only by the $Foxp3^+$ population.

4. Discussion

This study demonstrates that regulatory $CD8^+$ T cells can be induced from diabetogenic 8.3 transgenic NOD mice, which express the TCR-α and TCR-β of a diabetogenic H-$2K_d$-restricted β-cell cytotoxic $CD8^+$ T cell clone and promote diabetes [19]. Original NY8.3 $CD8^+$ cloned T cells cause severe insulitis and diabetes when cotransferred with NOD $CD4^+$ T cells [20]. The 8.3-NOD mice in our colony develop diabetes more rapidly than the original NOD mice, but the incidence of diabetes does not differ from that of NOD mice (75% among females aged 20 weeks and 30 weeks, respectively). Santamaria et al. reported that high avidity of 8.3 TCR T cells devel-

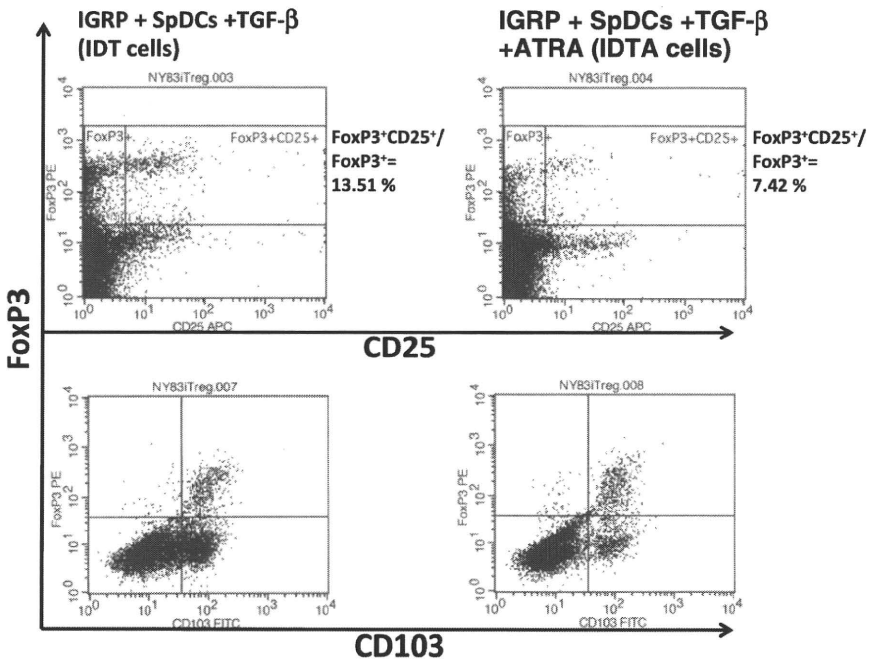

Fig. 2. IDT cells and IDTA cells were stained with antibodies against Foxp3, CD25, and CD103. Only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive. A representative example of four separate experiments is shown.

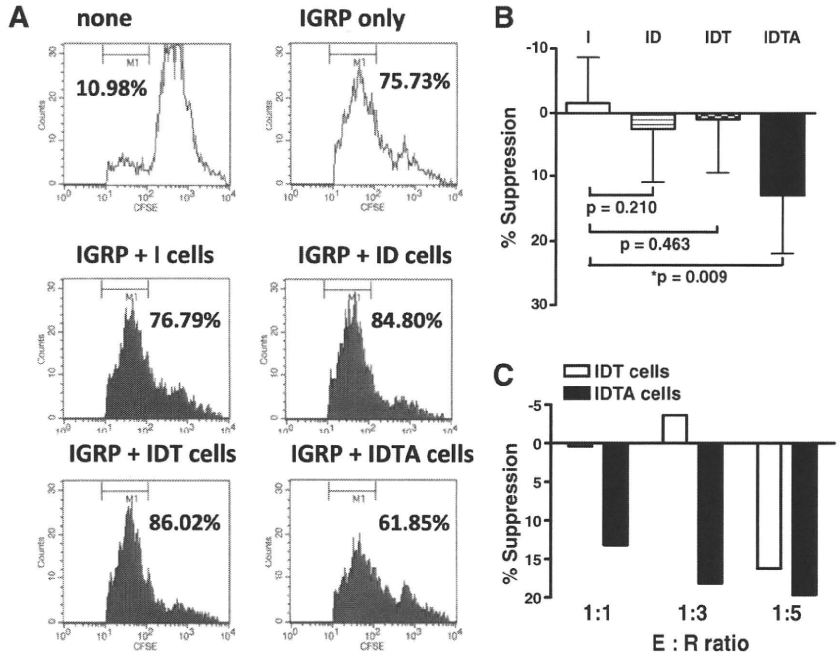

Fig. 3. CFSE-labeled $CD8^+$ T cells (1.0×10^5) purified from 8.3-NOD splenocytes were cultured for 5 days with IGRP alone or with IGRP and 1.0×10^5 induced $CD8^+$ T cells in each group shown in Fig. 1. Three days later, all cells were harvested and gated on $CFSE^+$ cells. (A) Proliferation of $CD8^+$ T cells induced by IGRP alone or in the presence of I, ID, IDT, or IDTA cells with IGRP was examined. A representative example of eight separate experiments is shown. (B) Proliferation of effector $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was shown as % suppression, in comparison with that induced by IGRP alone ($^*p = 0.009$, I vs. IDTA). (C) Proliferation of effector $CD8^+$ T cells (E) in the presence of IDT, or IDTA cells (R) with IGRP in comparison with that induced by IGRP alone was shown as % suppression at the indicated E:R ratios.

ops with aging in 8.3-NOD mice [21]. Because 10–20% of the mice did not develop diabetes, we presumed that regulatory T cells were present in 8.3-NOD mice. We generated Tregs in $CD8^+$ T cells for this reason.

Induced regulatory $CD8^+$ T cells have been used in some studies [14–17], two of which showed that Foxp3 is an important marker of regulatory $CD8^+$ T cells [14,17]. Regulatory $CD8^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA showed suppressor activity and the highest expression of Foxp3, which indicates that $CD8^+Foxp3^+$ T cells played a key role in the generation of regulatory $CD8^+$ T cells. Although the role of the expression of Foxp3 in these $CD8^+$ T cells is not well understood, Foxp3 expression is known to induce regulatory T cells. The Foxp3-transduced 6426 $CD8^+$ T cell clone, which recognizes insulin B chain peptide 15–23, delayed the

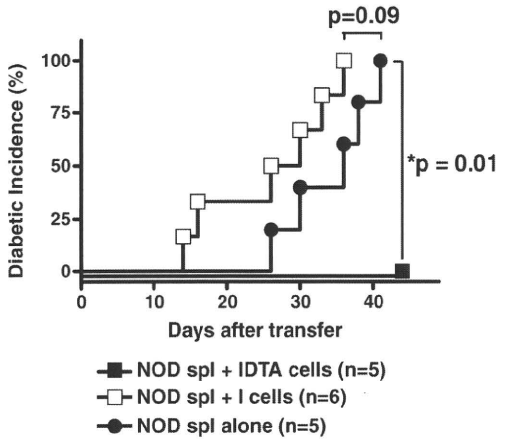

Fig. 4. Diabetogenic NOD splenocytes (1×10^7) alone (closed circle, $n = 5$) or with 6.0×10^6 I cells (open square, $n = 6$) or IDTA cells (closed square, $n = 5$) were transferred into 7- to 8-week-old NOD-scid mice. I cells had no effect on suppression of diabetes transfer, whereas IDTA cells completely suppressed diabetes transfer ($^*p = 0.01$, IDTA vs. control).

onset of diabetes compared with control 6426 clone when transferred into NOD-scid or young NOD mice [22]. This study indicated that expression of Foxp3 changes effector $CD8^+$ T cells into regulatory $CD8^+$ T cells.

Rigorous purification of regulatory $CD8^+Foxp3^+$ T cells in cellular transfusion material would prevent autoimmune diabetes. Because Foxp3 is not a surface marker, we examined whether the $CD8^+Foxp3^+$ T cells express specific surface markers that would enable purification of these cells *in vitro*. Unlike the case with $CD4^+CD25^+Foxp3^+$ T cells, CD25 is not a marker of regulatory $CD8^+$ T cells. More than 90% of the $CD8^+Foxp3^+$ T cells induced in our study also expressed CD103. CD103, the αEβ7 integrin, is a marker for alloantigen-induced regulatory $CD8^+$ T cells [15,23,24]. As $CD8^+Foxp3^-$ T cells also expressed CD103, it is not completely specific for $CD8^+Foxp3^+$ T cells. Purification of regulatory T cells using a CD103 antibody may isolate $CD8^+Foxp3^+$ T cells more efficiently.

ATRA has been reported to play an important role in immune system so far. It has been currently used to treat acute leukaemia and acne vulgaris [8,9]. The role of ATRA in the generation of $CD4^+$ regulatory T cells has also been reported recently [10–12]. Belkaid et al. reported that naïve $CD4^+Foxp3^-$ T cells converted $CD4^+Foxp3^+$ T cells in the gut and that gut-resident DCs produced ATRA. This conversion of Tregs occurred in a TGF-β and ATRA-dependent fashion [12]. Powrie et al. also reported that ATRA-dependent naïve T cells converted to Tregs after oral administration of antigen [11]. $CD4^+Foxp3^-$ T cells cultured with TGF-β, IL-2, and ATRA can convert into a $CD4^+Foxp3^+\alpha E\beta7^+CCR9^+$ phenotype *in vitro* and ATRA enhances the expression of Foxp3 and increases their suppressor activity [10]. ATRA can induce regulatory $CD4^+$ T cells from naïve $CD4^+$ T cells more efficiently in a Foxp3-dependent way. In this study, we first showed that ATRA and TGF-β was used to generate regulatory $CD8^+$ T cells *ex vivo*.

5. Conclusions

ATRA and TGF-β induce antigen-specific regulatory $CD8^+$ T cells in autoimmune diabetic mice. Regulatory $CD8^+$ T cells induced *ex vivo* would be useful as a therapeutic tool for autoimmune diabetes.

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LETTERS TO THE EDITOR

BIGUANIDE, BUT NOT THIAZOLIDINEDIONE, IMPROVED INSULIN RESISTANCE IN WERNER SYNDROME

To the Editor: Werner syndrome (WS) is an autosomal recessively inherited disorder and is known for adult progeria characterized by clinical phenotypes of early aging. The cause of this disease is identified as homologous mutations in WS protein (WRN), a RECQ family deoxyribonucleic acid (DNA) helicase gene.[1] In particular, WS patients often represent type 2 diabetes mellitus characterized by marked insulin resistance.

A 55-year-old female patient with WS, who recently died at 63 years of age, had visited our hospital for glycemic control. She was thin (body mass index 18.6 kg/m^2) and had the characteristic features: loss of hair, cataracts, skin ulcer, soft tissue atrophy, history of thyroid tumor, and type 2 diabetes mellitus. Genomic DNA analysis revealed that she was homozygote for type 6 (6/6) mutation in WRN DNA helicase gene, and WRN protein was not detected by immunoblot analysis.[2] Diabetic duration was 17 years, her having been diagnosed with diabetes mellitus at 38 years of age. Fasting serum C-reactive protein (CRP) and 24-hour urinary CRP were 2.8 ng/mL and 58.0 μg/d, respectively. These results showed that her insulin secretion was not impaired, suggesting the presence of insulin resistance. The number of insulin receptors and the insulin receptor tyrosine kinase activity in her erythrocytes were measured using enzyme-linked immunosorbent assay method and were found to be normal.[3] Although she had been treated with 42 U daily of humalin3/7 (insulin), blood glucose remained high, and her glycosylated hemoglobin (HbA1c) was 9.8%. Therefore, troglitazone (TRO, thiazolidinedione), a peroxisome proliferator-activated receptor-γ agonist, buformin (biguanide) on humalin3/7, or both were added. TRO administration on humalin3/7 failed to improve glycemic control. In contrast, buformin on humalin3/7 markedly improved glycemic control from 9.8% to 6.9% at HbA1c level and reduced daily insulin requirement from 42 U to 28 U. Moreover, additional TRO administration on buformin plus humanlin3/7 showed neither improvement of glycemic control nor reduction of daily insulin requirement. A euglycemic-hyperinsulinemic clamp was performed during insulin alone and buformin plus insulin therapy. Exogenous glucose infusion rate (GIR) at an insulin concentration of 150 μU/mL was 3.65 mg/kg per minute during insulin therapy alone and 3.68 mg/kg per minute during buformin plus insulin therapy. At an insulin concentration of 400 μU/mL, which suppresses hepatic glucose production, each GIR was similarly increased, up to approximately 11 mg/kg per minute (Figure 1). These results indicated that her insulin resistance depended on impairment of peripheral glucose uptake improved by high insulin concentration. In addition, there were no differences in GIR between the therapies with or without biguanide, but biguanide clinically improved her insulin resistance, suggesting the presence of another factor.

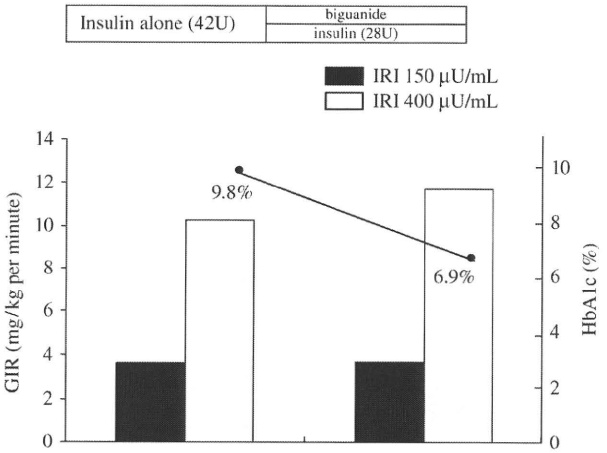

Figure 1. Change in glycosylated hemoglobin (HbA1c), final insulin requirement, and glucose infusion rate (GIR) in euglycemic-hyperinsulinemic clamp before and during biguanide plus insulin therapy.

Thiazolidinedione improves insulin sensitivity primarily by increasing glucose uptake and biguanide by decreasing glucose production.[4] A previous report demonstrated that biguanide activates adenosine monophosphate (AMP)-activated protein kinase as well as adiponectin, resulting in a decrease in hepatic glucose production.[5] In the current case, biguanide, but not thiazolidinedione, markedly improved insulin resistance in WS. Recently, biguanide and insulin have been reported to suppress hepatic gluconeogenesis through phosphorylation of cyclic AMP response element binding protein–binding protein through the different signaling pathways.[6] This is compatible with the findings of the current study that combination therapy of biguanide and insulin had an additive lowering effect on blood glucose levels.

This experience provides new evidence that an increase in endogenous glucose production rather than a decrease in peripheral glucose uptake could account for insulin resistance in WS and that biguanide may be a promising therapeutic tool for insulin resistance in this disease.

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THE MAN WHO COULDN'T CLOSE HIS MOUTH

To the Editor: An 82-year-old man was referred for a suspected transient ischemic attack. He said that, while having a meal, he was suddenly unable to close his mouth. He was unable to chew and continue his meal but was better after about 10 minutes. On further questioning, he also complained of aches in his jaw on chewing and intermittent pain in the neck associated with weakness. He had several similar episodes where he could not close his jaw since then, including an episode during the consultation.

Routine investigations including inflammatory markers were normal, excluding giant cell arteritis. His acetylcholine receptor antibody was positive at 43.1 (normal < 5). By this time, he had also developed intermittent dysphagia and generalized weakness. A diagnosis of myasthenia gravis was made, and all his symptoms resolved after intravenous immunoglobulins and oral pyridostigmine and prednisolone.

Myasthenia gravis is largely underdiagnosed in the elderly population and can present with subtle and unusual clinical patterns.[1,2] Acetylcholine receptor antibody, muscle-specific tyrosine kinase antibody, and repetitive nerve stimulation studies usually confirm the diagnosis.[3,4] It should always be remembered to maintain a high index of clinical suspicion in elderly patients with subtle neurological and musculoskeletal symptoms, as in this case, especially given that the prevalence of myasthenia gravis in elderly people has increased.[5,6]

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Sponsor's Role: None.

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ACUTE UPPER GASTROINTESTINAL BLEEDING IN ELDERLY PEOPLE: PRESENTATIONS, ENDOSCOPIC FINDINGS, AND OUTCOMES

To the Editor: Acute upper gastrointestinal (GI) bleeding in elderly patients is a commonly encountered medical problem, with annual hospitalization costs estimated to be approximately $1.48 billion.[1] The rate of admission for acute upper GI bleeding increases 30-fold between the third and ninth decades of age, and the percentage of patients aged 60 and older with a diagnosis of acute upper GI bleeding increased from 46.1% in 1987 to 63.2% in 2001.[2,3]

The increase in the incidence of acute upper GI bleeding in elderly people has been attributed to many factors, including an increase in the use of nonsteroidal anti-inflammatory drugs (NSAIDs) in elderly people,[3]who are at greater risk of GI toxicity from these agents, as well as a higher prevalences of *Helicobacter pylori*[4] and gastroesophageal reflux disease in elderly people.[3,4]

Several studies in the GI literature have compared acute upper GI bleeding in the elderly population with that in younger individuals. The incidence of acute upper GI bleeding was more common in women than men.[5–7] Peptic ulcer disease is the most common source of acute upper GI bleeding in elderly people.[8–10] Esophagitis is a common etiology for acute upper GI bleeding, especially in patients aged 80 and older.[5,9] Variceal bleeding is more commonly seen in younger patients than in those aged 75 and older.[11]

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Age-Associated Increase in Abdominal Obesity and Insulin Resistance, and Usefulness of AHA/NHLBI Definition of Metabolic Syndrome for Predicting Cardiovascular Disease in Japanese Elderly with Type 2 Diabetes Mellitus

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Key Words
Diabetes · Obesity · Waist circumference · Insulin resistance · Metabolic disease clustering

Abstract
Background: Management of metabolic syndrome (MetS) seems to constitute an efficient strategy to attain successful ageing. Although the clinical entity of MetS in patients with diabetes mellitus has been discussed, there is very little information on MetS-type cardiometabolic risk factor clustering in diabetic elderly. ***Objective:*** To determine the relationship among age-associated changes in obesity, insulin resistance, and clustering of MetS-type risk factors, in association with vascular complications, in Japanese elderly with type 2 diabetes. ***Methods:*** A cross-sectional study was conducted of 812 diabetic elderly enrolled in the Japanese Elderly Diabetes Intervention Trial. Information on diabetes, blood examinations and complications was obtained. Abdominal obesity, insulin resistance and prevalence of MetS risk factor clustering, defined by three sets of criteria from the International Diabetes Federation (IDF), the Japanese Society of Internal Medicine (JSIM), and the American Heart Association and the National Heart, Lung, and Blood Institute (AHA/NHLBI), were analyzed. ***Results:*** Waist circumference and insulin resistance estimated by homeostasis model assessment insulin resistance (HOMA-IR) increased with age, followed by a partial decrease at age 80 and over. Prevalence of IDF-MetS and JSIM-MetS also increased with age at least until the age of 80, whereas the incidence of AHA/NHLBI-MetS did not show any apparent age changes. There was a significant crude linear association between waist circumference and HOMA-IR, which was highly elevated in IDF and AHA/NHLBI overlapping with MetS, and also elevated in AHA/NHLBI without abdominal obesity. Although IDF-MetS and JSIM-MetS, which specify abdominal obesity, did not always appear to be associated with cardiovascular diseases, AHA/NHLBI-MetS, comprising both abdominal obesity and non-abdominal obesity, independently correlated with coronary heart disease and stroke after adjustment for other risk factors of atherosclerotic diseases. ***Conclusion:*** There was an age-associated increase in the prevalence of abdominal obesity and insulin resistance in elderly diabetic Japanese subjects, with a clear relationship between waist circumference and insulin resistance. However, insulin resistance was elevated not only in cases with but also in those

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without abdominal obesity if accompanied by clustering of metabolic disorders. The AHA/NHLBI definition of MetS proved to be the most useful to predict cardiovascular disease in the diabetic elderly.

Introduction

Metabolic syndrome (MetS) consists of multiple, interrelated risk factors of metabolic origin that appear to directly promote the development of cardiovascular disease. Recently, MetS has been reported to be associated with impaired activities of daily living (ADLs) and cognitive decline of the elderly [1, 2]. Thus, management of risk factors and prevention of MetS seem to constitute an efficient strategy to attain successful ageing. Although insulin resistance and visceral adiposity could play a pivotal role in promoting atherosclerosis, the real cause may be a more complex interaction between genetic and environmental factors [3, 4].

The clinical usefulness of MetS for risk prediction for individuals with type 2 diabetes remains a matter of debate. It has been reported that insulin resistance and MetS are predictive of accelerated atherosclerosis in type 2 diabetic patients [5–14]. On the other hand, a recent reappraisal of MetS endorsed by the American Diabetes Association and the European Association for the Study of Diabetes argues that MetS is an entity of little or no prognostic use for diabetic patients [15]. They emphasized that it remains unclear whether identification of MetS confers a clinical advantage over identification and treatment of its individual components. Although the term MetS may not be applicable to diabetic subjects, even detractors agree that there are diabetic elderly who have increased insulin resistance associated with MetS-type cardiometabolic risk factor clustering [5–14].

Moreover, ageing is associated with increased insulin resistance in addition to type 2 diabetes [16]. Thus, the impact of abdominal obesity on insulin resistance and clustering of metabolic risk factors in diabetic elderly remains unknown. To date, for Asian elderly with type 2 diabetes, there is limited information on age-associated changes in abdominal obesity, insulin resistance and clustering of metabolic risk factors, and their association with vascular complications.

To address the need for elucidation concerning metabolic risk factor clustering in diabetic elderly, we conducted a large-scale prospective study of the Japanese Elderly Diabetes Intervention Trial (J-EDIT) [17, 18]. The questions we addressed were: (1) prevalence of abdominal obesity, insulin resistance, and MetS-type risk factor clustering as defined by different sets of criteria; (2) possible connections between abdominal obesity and insulin resistance, and (3) the predictive power of MetS-type clustering of metabolic risk factors for cardiovascular diseases in diabetic elderly. To answer these questions, we analyzed the baseline measures of the J-EDIT.

Methods

Participants

J-EDIT started in 2001 with an enrolment of 1,173 diabetic subjects aged 65 years or over and with serum HbA1c levels of ≥7.0% from 42 institutes in Japan. The J-EDIT protocol, which is in accordance with the provisions of the Declaration of Helsinki, received ethical approval from the institutional review boards of all of the participating institutes. Written informed consent was obtained from all patients. All examinations relevant for this study were completed by 812 subjects, 371 of whom were men. The remaining 361 subjects were excluded because some of their data were missing.

Diagnostic Criteria for MetS

In this study, we applied the three different sets of criteria proposed for the diagnosis of MetS by the International Diabetes Federation (IDF), the Japanese Society of Internal Medicine (JSIM) and the American Heart Association and the National Heart, Lung, and Blood Institute (AHA/NHLBI) [19–21]. According to IDF and JSIM criteria, there is a strong correlation between abdominal obesity and insulin resistance, which makes the presence of abdominal obesity a condition for diagnosis of MetS [19, 20]. A Japanese study concluded that a visceral fat area in excess of 100 cm^2 measured by means of CT scanning corresponds to a waist circumference of 85 cm for men and ≥90 cm for women [22]. In 2005, moreover, the IDF recognized specific cutoffs by sex and ethnicity [23]. However, new data support the use of alternative waist circumference cutoffs for the prediction of cardiovascular complications [24–26]. In 2007, the IDF recommended new waist circumference cutoffs for Japanese, 90 cm for men and 80 cm for women [19]. However, these cutoffs for waist circumference in the definition of MetS for Japanese have remained a matter of debate. For our study, we therefore adopted two criteria, one from the IDF (2007) and one from the JSIM. On the other hand, AHA/NHLBI has introduced alternative criteria, which have the advantage of avoiding emphasis on a single cause [21]. The resulting three definitions for MetS are as follows.

(1) The IDF definition of MetS (IDF-MetS) specifies abdominal obesity with waist circumference cutoffs of ≥90 cm for men or ≥80 cm for women plus any one of the following factors [19]: (a) elevated triglyceride (≥150 mg/dl) or specific treatment for this lipid abnormality; (b) reduced HDL-cholesterol of <40 mg/dl for men or <50 mg/dl for women, and (c) elevated systolic blood pressure (≥130 mm Hg) or diastolic blood pressure (≥85 mm Hg) or treatment for previously diagnosed hypertension.

Table 1. Demographic and clinical parameters of the patients

	Male	Female	All subjects
n	371	441	812
Age, years	71.4 ± 4.5	72.0 ± 4.6	71.8 ± 4.6
Duration of diabetes, years	16.7 ± 10.2	15.7 ± 8.8	16.2 ± 9.5
Systolic blood pressure, mm Hg	137.1 ± 15.8	138.6 ± 17.3	137.9 ± 16.7
Diastolic blood pressure, mm Hg	76.2 ± 9.6	75.5 ± 10.0	75.8 ± 9.9
HbA1c, %	8.0 ± 0.9	8.0 ± 0.9	8.0 ± 0.9
Fasting plasma glucose, mg/dl	169.7 ± 50.5	165.1 ± 50.7	167.2 ± 50.6
Fasting plasma insulin, μU/ml	9.3 ± 10.0	10.2 ± 8.5	9.8 ± 9.3
Urine Alb, mg/g Cr	226.4 ± 597.9	184.5 ± 499.7	203.7 ± 546.8
Serum cholesterol, mg/dl	192.7 ± 31.2	209.5 ± 34.5	201.8 ± 34.1
Serum HDL-C, mg/dl	52.9 ± 15.5	60.3 ± 19.4	56.9 ± 18.1
Serum LDL-C, mg/dl	114.9 ± 28.0	123.7 ± 32.0	119.7 ± 30.5
Serum triglyceride, mg/dl	129.2 ± 84.1	129.4 ± 66.4	129.3 ± 74.9
Current smokers, %	28.4	5.8	16.2
OHA use, %	62.5	61.7	62.1
Insulin use, %	25.3	31.1	28.5
Medication for hypertension, %	47.3	61.9	55.1
Medication with fibrates, %	3.2	4.3	3.8
Medication with statin, %	18.9	43.1	32.0
History of CHD, %	15.5	15.6	15.6
History of stroke, %	14.7	11.3	12.9

Data are presented as means ± SD or as percentages. OHA = Oral antihyperglycemic agents; CHD = coronary heart disease.

(2) The JSIM definition of MetS (JSIM-MetS) specifies abdominal obesity with waist circumference cutoffs of ≥85 cm for men or ≥90 cm for women, plus any one of the following factors [20]: (a) elevated triglyceride (≥150 mg/dl) or reduced HDL-cholesterol (<40 mg/dl) or specific treatment for these lipid abnormalities, and (b) elevated systolic blood pressure (≥130 mm Hg) or diastolic blood pressure (≥85 mm Hg) or treatment for previously diagnosed hypertension.

(3) The AHA/NHLBI definition of MetS (AHA/NHLBI-MetS) specifies two or more of the following conditions [21]: (a) waist circumference of ≥90 cm for men or ≥80 cm for women; (b) elevated triglyceride (≥150 mg/dl) or specific treatment for lipid abnormality; (c) reduced HDL-cholesterol of <40 mmol/l for men or 50 mmol/l for women, and (d) elevated systolic blood pressure (≥130 mm Hg) or diastolic blood pressure (≥85 mm Hg) or treatment for previously diagnosed hypertension.

Assessment of Diabetes Mellitus and Complications

Information about diabetes mellitus, blood examinations and complications were obtained from clinical charts. Waist circumference was measured at the umbilicus level. Information regarding cigarette smoking was collected using a standardized questionnaire.

After overnight fasting, blood samples were taken by vein puncture to assess serum levels of glucose, HbA1c, total cholesterol, triglyceride, and HDL-cholesterol. Insulin resistance was assessed from levels of fasting glucose and insulin concentration by means of the homeostasis model assessment (HOMA) formula: fasting insulin (μU/ml) × fasting glucose (mg/dl)/405 [27]. This method was not applicable to subjects treated with insulin. Serum LDL-cholesterol levels were calculated using Friedewald's equation, except for triglyceride levels of >400 mg/dl, in which case the LDL cholesterol data were recorded as 'missing'.

Information about a previous history of coronary heart disease (CHD) and stroke and findings from a 12-lead electrocardiogram (ECG) were obtained for all patients to assess cardiovascular disease at baseline. CHD was considered to be present when diabetic patients had at least one of the following: a history of myocardial infarction and angina characterized by a typical clinical picture (chest pain, chest oppression, dyspnea, typical ECG alteration). Stroke events were defined as a constellation of neurological deficits of sudden or rapid onset for which there was no apparent cause other than a vascular accident. Cases with asymptomatic lesions detected by brain imaging were not included.

Statistical Analysis

Data are presented as means ± SD or as percentages unless otherwise specified. Association of waist circumference with HOMA insulin resistance (HOMA-IR) was tested using simple and multiple logistic regression. Variables among the MetS subgroups were compared using ANOVA and statistical differences were tested with Dunnett's statistical test. Backward logistic regression analysis was used to calculate the adjusted odds ratio (OR) and 95% confidence interval (CI) for risk factors with cardiovascular diseases. The SAS software package (Version 8.0; SAS, Cary, N.C., USA) was used for all analyses. $p < 0.05$ was considered significant.

Table 2. Age-associated changes in BMI, waist circumference, HOMA-IR, and prevalence of MetS risk factors

	Men, age group				Women, age group			
	65–69	70–74	75–79	80–85	65–69	70–74	75–79	80–85
BMI	23.8 ± 3.2	23.9 ± 3.1	23.9 ± 3.1	23.6 ± 2.7	23.6 ± 3.5	24.1 ± 3.8	24.5 ± 3.7	22.8 ± 3.3
Waist circumference, cm	85.8 ± 8.9	85.0 ± 8.1	87.6 ± 8.1	88.3 ± 10.0	80.6 ± 10.4	82.7 ± 10.7	84.5 ± 11.2	79.6 ± 10.2
HOMA-IR	3.81 ± 4.0	3.37 ± 3.1	4.37 ± 5.8	2.83 ± 2.0	3.17 ± 2.7	3.49 ± 3.0	4.80 ± 3.6	3.54 ± 4.1
IDF-MetS, %	28.6	30.4	33.3	42.1	47.9	59.6	59.6	46.9
JSIM-MetS, %	51.7	47.4	59.7	68.4	19.4	23.0	31.7	25.0
AHA/NHLBI-MetS, %	56.5	52.6	59.7	57.9	72.2	83.2	80.8	65.6
Hypertension, %	79.6	74.6	84.5	84.2	77.8	87.6	91.3	96.9
IDF and AHA/NHLBI								
Low HDL-C, %	38.1	30.6	35.2	31.6	60.4	65.8	65.4	46.9
High triglyceride, %	26.5	30.6	25.4	36.8	29.2	36.6	30.8	25.0
JSIM dyslipidemia, %	36.1	37.3	33.8	47.4	30.6	39.1	32.7	28.1

Data are presented as means ± SD or as percentages. BMI = Body mass index; HOMA-IR = homeostasis model assessment, insulin resistance; MetS = metabolic syndrome; IDF = International Diabetes Federation; JSIM = Japanese Society of Internal Medicine; AHA/NHLBI = American Heart Association and the National Heart, Lung, and Blood Institute.

Results

Age-Associated Increase in Abdominal Obesity, Insulin Resistance and MetS-Type Risk Factor Clustering

Demographic and clinical parameters of the 812 study participants are listed in table 1. Age-associated changes in BMI, waist circumference, and HOMA-IR are listed in table 2. BMI did not show any apparent changes for men, although waist circumference increased with age. Waist circumference also increased for women, followed by a decrease at the age of 80 or over. HOMA-IR similarly increased with age, but decreased from the age of 80 for both men and women. The increase in insulin resistance seemed to correlate with the age-associated increase in abdominal obesity of diabetic elderly.

The overall prevalence of MetS-type risk factor clustering based on IDF, JSIM and AHA/NHLBI criteria was 44.0, 37.1 and 67.7%, respectively. The incidence of IDF-MetS and JSIM-MetS, which specify abdominal obesity, increased with age, but decreased at age 80 or over for women (table 2). In contrast, the prevalence of AHA/NHLBI-MetS did not show any apparent change with ageing.

As for the individual components of MetS, the prevalence of hypertension was highest and increased with age. Among diabetic elderly aged 80–85 years, hypertension was found in 84.2% of men and 96.9% of women. The prevalence of low HDL-cholesterol also increased with age, but started to decrease at the age of 80 and over.

We also investigated whether diabetic patients with a longer history of diabetes and/or more serious hyperglycemia might show an increased prevalence of MetS-type risk factor clustering, but no such trend could be found in any type of criteria-defined MetS (data not shown).

Possible Connections between Abdominal Obesity and Insulin Resistance

Overall, HOMA-IR was 4.2 ± 5.0 for diabetic elderly. The relationship between waist circumference and insulin resistance is shown in figure 1. Simple regression analysis showed that log-transformed HOMA-IR was associated with waist circumference in a crude linear manner (coefficient = 0.051, $p < 0.0001$, $R^2 = 0.105$ for men; coefficient = 0.034, $p < 0.0001$, $R^2 = 0.116$ for women). This was demonstrated by an increase in HOMA-IR of 1.1 for men and 0.6 for women for every 10-cm increment in waist circumference. After adjustment for sex, age, systolic blood pressure, HbA1c, triglyceride, and HDL-C, the association remained statistically significant (coefficient = 0.034, $p < 0.0001$).

For any of the three definitions, HOMA-IR was higher for subjects with MetS than for those without MetS (4.34 ± 3.65 for IDF-MetS and 3.21 ± 3.50 for non-IDF-MetS, p = 0.0003; 4.33 ± 3.95 for JSIM-MetS and 3.30 ± 3.33 for non-JSIM-MetS, p = 0.0022; 4.03 ± 3.58 for AHA/NHLBI-MetS and 3.04 ± 3.57 for non-AHA/NHLBI-MetS, p = 0.0022).

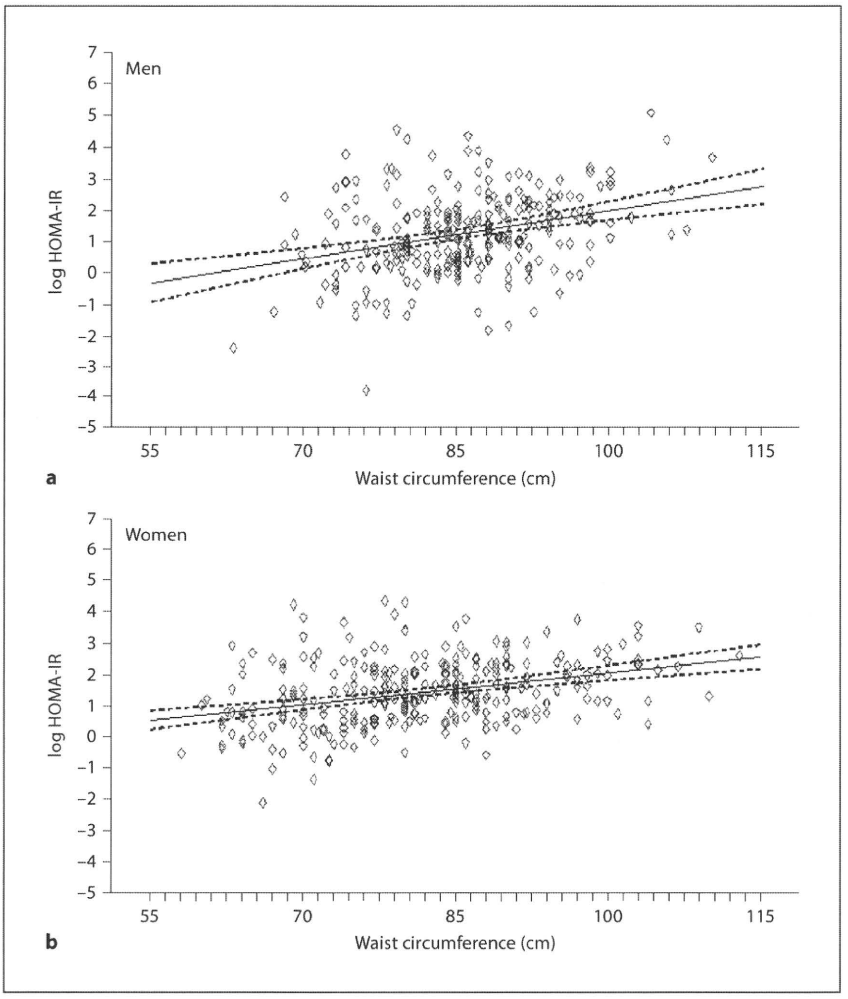

Fig. 1. Relationship between waist circumference and insulin resistance. Association of HOMA-IR with waist circumference of men (**a**) and women (**b**) in the J-EDIT. Log-transformed HOMA-IR (log HOMA-IR) was associated with waist circumference in a crude linear manner (coefficient = 0.051, $p < 0.0001$, $R^2 = 0.105$ for men; coefficient = 0.034, $p < 0.0001$, $R^2 = 0.116$ for women).

It has been proposed that HOMA-IR is useful for estimating insulin resistance of type 2 diabetic patients [28], but the degree of association between HOMA-IR and clamp insulin resistance for diabetic patients treated with oral antihyperglycemic agents of the class insulin secretagogues has remained unclear. In this connection, Emoto et al. [29] have reported that HOMA-IR strongly correlates with clamp insulin resistance in type 2 diabetic patients treated with sulfonylureas (SUs) as well as in those treated with diet alone. Furthermore, Spearman's correlation coefficients for HOMA-IR and waist circumference were similar for subjects taking SU drugs and those who had used neither SU drugs nor glinides (data not shown). Such evidence indicates that it seems likely that waist circumference is associated with insulin resistance in diabetic elderly, regardless of treatment with oral antihyperglycemic agents of the class insulin secretagogues.

Insulin Resistance of Metabolic Factor Clustering with and without Abdominal Obesity

We compared the clinical characteristics of IDF-MetS and AHA/NHLBI-MetS by dividing the study population into 3 subgroups, non-MetS, AHA/NHLBI-only, and IDF&AHA/NHLBI (table 3). There was no difference in age among the subgroups. HOMA-IR was especially elevated in the IDF&AHA/NHLBI group, as was waist circumference. Interestingly, in the AHA/NHLBI-only group, HOMA-IR was moderately elevated without an accompanying increase in waist circumference. Furthermore, the mean duration of diabetes for MetS with overlapping patterns was significantly shorter than that for

Table 3. Clinical characteristics of subgroups of IDF-MetS and AHA/NHLBI-MetS risk factor clustering

	Non-MetS	AHA/NHLBI only	IDF&AHA/NHLBI
Number	263	192	357
Age, years	71.9 ± 4.7	71.0 ± 4.5	72.1 ± 4.5
Waist circumference, cm	78.2 ± 7.9	77.7 ± 6.8	91.4 ± 7.3*
HOMA-IR	3.04 ± 3.6	3.46 ± 3.4	4.34 ± 3.7*
Duration of diabetes, years	16.1 ± 10.3	14.0 ± 8.2	13.6 ± 8.7‡

For abbreviations see table 2. Data are means ± SD or actual numbers.

* $p < 0.001$, † $p = 0.004$, ‡ $p = 0.012$, in comparison with non-MetS.

Table 4. Association of MetS and other risk factors with cardiovascular disease

Previous history of CHD			Previous history of stroke		
	OR	95% CI		OR	95% CI
IDF					
MetS	1.44	0.91–2.28	MetS	1.16	0.70–1.92
Sex	1.39	0.88–2.19	Sex	1.69	1.03–2.75
Age	1.06	1.01–1.12	Age	1.05	0.99–1.10
DBP	0.98	0.95–0.99	TG	1.01	1.00–1.01
			DM duration	1.25	0.89–1.75
JSIM					
MetS	1.80	1.14–2.85	MetS	1.32	0.79–2.19
Sex	1.10	0.70–1.73	Sex	1.51	0.92–2.47
Age	1.06	1.01–1.11	Age	1.05	0.99–1.10
DBP	0.97	0.95–0.99	TG	1.01	1.00–1.01
			DM duration	1.27	0.90–1.78
AHA/NHLBI					
MetS	1.90	1.13–3.19	MetS	1.86	1.07–3.24
Sex	1.48	0.94–2.33	Sex	1.84	1.13–3.00
Age	1.06	1.01–1.12	Age	1.05	0.99–1.11
DBP	0.97	0.95–0.99			
Factors other than MetS					
Sex	1.26	0.82–1.95	Sex	1.63	1.01–2.61
Age	1.01	1.01–1.12	Age	1.05	0.99–1.11
DBP	0.98	0.96–0.99	TG	1.01	1.00–1.01
			DM duration	1.25	0.89–1.75

MetS = Metabolic syndrome; CHD = coronary heart disease; SBP = systolic blood pressure; DBP = diastolic blood pressure; TG = triglyceride; DM = diabetes mellitus. For other abbreviations, see table 2.

The association of IDF-MetS, JSIM-MetS and AHA/NHLBI-MetS with cardiovascular disease was examined. MetS and sex were included in the independent variables, and age, HbA1c, duration of diabetes, smoking, total cholesterol, LDL-cholesterol, TG, SBP and DBP were analyzed with backward stepwise regression.

non-MetS. These results suggest that there are two distinct ways for insulin resistance to increase in diabetic elderly, one is in association with abdominal obesity and the other is not relevant to abdominal obesity.

Association of MetS-Type Risk Factor Clustering with Cardiovascular Disease

We examined the independent association of MetS-type risk factor clustering with cardiovascular diseases (table 4). Because sex is reportedly an independent factor associated with MetS [30–32], MetS and sex were included in the independent variables, while the other risk factors for atherosclerotic disease, such as age, HbA1c, duration of diabetes, smoking, total cholesterol, LDL-cholesterol, triglyceride, systolic blood pressure and diastolic blood pressure, were analyzed with backward stepwise regression. Age was found to be consistently associated with CHD, while JSIM-MetS and AHA/NHLBI-MetS, but not IDF-MetS, were also associated with CHD. When MetS was eliminated from the independent variables, age and diastolic blood pressure proved to be significantly associated with CHD, suggesting these factors independently correlate with CHD in diabetic elderly. For stroke, AHA/NHLBI-MetS was identified as a predictive factor. When MetS was eliminated from the independent variables, sex (men) and triglyceride showed a significant correlation with stroke. On the other hand, IDF-MetS and JSIM-MetS, which both specify the presence of abdominal obesity for MetS, were not associated with stroke. These results indicate that MetS of AHA/NHLBI definition is the most consistent predictor for CHD and stroke for diabetic elderly, even after adjustment for the risk factors of age, sex, blood pressure, dyslipidemia, and indices of diabetes.

Discussion

This J-EDIT study first provided evidence of MetS-type risk factor clustering in Asian (Japanese) elderly with type 2 diabetes. Several new findings are reported: (1) abdominal obesity, insulin resistance and prevalence of MetS-type risk factor clustering evidently increased with age, but somewhat decreased at the age of 80 and over; (2) overall insulin resistance was substantially elevated in diabetic elderly [28, 30], and there was a significant crude linear association between waist circumference and insulin resistance; (3) insulin resistance was elevated not only in cases with but also without abdominal

obesity if accompanied by clustering of metabolic disorders, and (4) AHA/NHLBI-MetS, comprising both abdominal obese and non-abdominal obese metabolic factor clustering cases, was the most useful for prediction of cardiovascular disease in diabetic elderly.

The incidence of MetS in the general population reportedly differs widely among ethnic groups and according to the definition of MetS [13, 33–38]. It has also been reported that the prevalence of MetS increases with age [39, 40]. However, the prevalence of MetS-type risk factor clustering among patients with known diabetes is consistently high regardless of ethnicity or definition [6–13, 30, 33, 41–48]. In the Japan Diabetes Complications Studies (JDCS), which was concerned with relatively younger diabetic patients aged 40–70 years, the prevalence of IDF-MetS risk factor clustering was 32% for men and 9.2% for women [47]. Although the inclusion criteria of JDCS and JEDIT were not the same, it seems likely that prevalence of MetS-type risk factor clustering in Japanese patients with type 2 diabetes increases with age, at least until the age of 80. To our knowledge, there are no epidemiological data of MetS-type risk factor clustering of diabetic elderly in the other ethnic groups.

Although we did not measure insulin resistance directly in this study, HOMA-IR has been shown to correlate well with direct methods in subjects with various degrees of glucose tolerance, including patients who have already developed diabetes [27]. The averages of HOMA-IR of younger diabetic subjects have been reported as 2.9–3.3 [28, 30]. In our diabetic elderly, insulin resistance was evidently high (4.2 ± 5.0), which may be due to the diabetic state itself and/or age-associated changes in body composition such as increases in fat mass and decreases in fat-free mass [16]. We therefore expected that the correlation of insulin resistance with abdominal obesity might become weaker in diabetic elderly, but there was in fact a significant linear association of insulin resistance with waist circumference, and the former was found to be higher in JSIM-MetS and IDF-MetS. In this respect, it should be pointed out that insulin resistance also increased moderately in MetS-type risk factor clustering without abdominal obesity, so that the mechanism for the increase in insulin resistance associated with non-obese type metabolic factor clustering remains to be clarified [40].

Evidence is accumulating that MetS is clinically relevant for the prediction of cardiovascular disease in non-diabetic elderly [49–51]. Our study is the first to demonstrate that AHA/NHLBI-MetS correlates independently with cardiovascular disease in diabetic elderly after adjustment for the other risk factors for atherosclerotic disease. It seems plausible that non-obese metabolic factor clustering together with increased insulin resistance has a major impact on the risk of cardiovascular diseases of diabetic elderly, because MetS with abdominal obesity does not always appear to be associated with cardiovascular diseases [10, 52–54]. Definitions of MetS-type risk factor clustering that specify abdominal obesity have not yet been developed for Asian (Japanese) diabetic elderly. Other studies have also identified the usefulness of the National Cholesterol Education Program (NCEP)-MetS and AHA/NHLBI-MetS for the prediction of cardiovascular disease in younger subjects with type 2 diabetes [9–10, 13]. On the other hand, Sone et al. [30] have demonstrated that NCEP-MetS has limited clinical usefulness as a predictor for Asian diabetic patients. Further prospective analyses are thus needed to investigate the clinical significance of MetS-type risk factor clustering without abdominal obesity for diabetic elderly.

There are certain limitations to our study. First, we performed a cross-sectional evaluation and our results are therefore subject to survival bias. Second, our study subjects were hospital-based patients with diabetes of relatively long duration, so that any inferences are of necessity limited to similar patient groups. On the other hand, this population sample represents the real-world scenario of type 2 diabetes in Japan.

In conclusion, abdominal obesity and insulin resistance were found to increase with age, at least until the age of 80, in Asian diabetic elderly, and a relationship between waist circumference and HOMA-IR was demonstrated. An important finding was that MetS-type metabolic factor clustering without abdominal obesity also showed elevated insulin resistance. AHA/NHLBI-MetS, comprising both obese and non-obese metabolic disease clustering, was found to be the most effective for the prediction of cardiovascular disease, whilst the significance of MetS with abdominal obesity in this respect remains unclear. An on-going prospective study of J-EDIT may help to clarify the pathophysiology of metabolic disease clustering and its association with cardiovascular disease and geriatric syndromes of diabetic elderly.

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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Association between masticatory performance and anthropometric measurements and nutritional status in the elderly

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Aim: The association between chewing ability and physical constitution and nutritional status remains uncertain in the elderly. We examined the relationships between chewing ability and anthropometric measurements or nutritional status in the elderly.

Methods: A total of 200 subjects (78 men and 122 women; mean age ± standard deviation, 76.6 ± 7.1) were enrolled from geriatric clinical settings. Chewing ability was evaluated by color-changeable chewing gum. Bodyweight, body mass index, mid-upper-arm circumference (MAC), and triceps skinfold, grip strength, serum albumin, physical and cognitive functions, depressive status, and dental status were determined.

Results: Correlations were found between chewing ability and bodyweight, MAC, dental status, physical and cognitive functions, and depressive status after adjusting for age and sex. The concentrations of serum albumin were well-correlated with chewing ability and anthropometric measurements. Stepwise linear regression analyses revealed that the masticatory cycle, dental status, bodyweight and MAC are predictors of chewing ability, and that age, chewing ability, grip strength and sex are predictors of serum albumin concentrations.

Conclusion: Chewing ability is associated with not only oral health status but also the physical constitution of the elderly. In addition, chewing ability may add to the regulation of the nutritional status in the elderly. **Geriatr Gerontol Int 2010; 10: 56–63.**

Keywords: albumin, anthropometry, chewing, mastication, nutrition.

Introduction

Mastication is the initial step in the digestive process, and is well known to be influenced by oral health status.[1,2] The oral health of older people has been intensively studied in the past decade, and the results suggest that oral comorbidities are common in older patients and that poor oral health not only plays an important role in quality of life, but also can contribute to serious morbidity and mortality in older patients.[3–7] However, much less attention has been paid to the impact of masticatory performance on the nutritional status and health of the elderly. This omission can partly be ascribed to the fact that masticatory function is a complex process and that its role in deglutition and digestion is difficult to judge.

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Author contributions: K. O. performed analysis and interpretation of data, and preparation of manuscript; H. E. performed statistical analysis and interpretation of data; S. I. acquired of data; A. I. performed study supervision; M. K. was responsible for study concept, design, conduct of study, interpretation of data and study supervision.

A variety of methods for measuring chewing ability are employed: self-assessed masticatory,[8] dentition status, especially the number of natural teeth and the use of dentures,[4] the number of occlusal contacts, including both teeth and dentures,[9] and use of an index consisting of six indicator foods.[10] Although objective methods seem to be more accurate, most of them are time-consuming and require specific facilities, making them expensive and cumbersome for use in epidemiological surveys. Among the various kinds of objective assessments, color-changeable chewing gum has been reported to be a useful test material for evaluating objective chewing ability.[11–13] However, it remains unknown whether chewing ability evaluated by this unique method is related to physical function, anthropometry and nutritional status in the elderly.

In this study, we tried to examine the relationships between the chewing ability of Japanese elderly, as assessed by the color-changeable chewing gum and various other factors, including anthropometric measurements, dental status, physical and cognitive functions, moods, and biochemicals, as nutritional parameters. Furthermore, we also examined whether chewing ability is associated with nutritional status in the elderly, using serum albumin levels as a nutritional parameter.

Methods

Subjects

All participants, 200 elderly people (78 men and 122 women; mean age ± standard deviation [SD], 76.6 ± 7.1 years; age range, 60–94 years), were consecutively enrolled from our geriatric outpatient clinic ($n = 96$) and from convalescent rehabilitation hospital ($n = 104$). Subjects diagnosed with infection, inflammation, liver disorders, kidney disorders, cancer or bone marrow proliferative disorders were excluded by the physicians. All participants provided written informed consent. The study was approved by the Institutional Review Board of the Nagoya University of Arts and Sciences at Nisshin City, Aichi.

Anthropometric measurements

Body mass index (BMI) is defined as the weight in kilograms divided by height in meters squared. Triceps skinfold (TSF, cm) was measured with Harpenden calipers over the triceps muscle at the midway point between the acromion and the olecranon process. Mid-upper-arm circumference (MAC, cm) was measured on the left arm with a tape measure. Three repeat measurements were taken to the nearest 0.5 mm, with the mean taken as the true value. Upper arm muscle area (AMA, cm^2) was calculated using the formula shown below: $AMA\ (cm^2) = (MAC - [TSF \times \pi]/10)^2/4\pi$.

Evaluation of chewing ability

All gum samples were kindly provided by Lotte (Tokyo, Japan). It has been demonstrated that this non-abrasive color-changeable gum test generates reliable and reproducible quantitative data and that there is a strong correlation between the color change and masticatory performance, suggesting that the color-changeable chewing gum is useful for measuring masticatory ability.[13] Subjects were asked to chew the non-abrasive color-changeable chewing gum for 1 min, as they usually chew. The number of chewing strokes was also measured during a 1-min test period. Immediately after the trials, the color of the gum samples was evaluated in a L* a* b* color system with a Spectrophotometer CM-2500d (Konica Minolta Sensing, Osaka, Japan). The L* a* b* color system was developed by the CIE (Commission Internationale de l' Eclairage) for measuring perceived color and color differences. The three basic coordinates represent the lightness of the color (L*), its position between red/magenta and green (a*), and its position between yellow and blue (b*). In this study, a* values were used to represent the masticatory ability, because it has been demonstrated that higher a* values are well-correlated with chewing performance.[11,13] Before the gum test, we examined the number of natural teeth, prostheses and occluding pairs of teeth using Eichner's index.[14] Eichner's index is based on the existence of occlusal contacts of the premolars and the molars, which is called supporting zones. A maximum of four supporting zones can exist where at least one tooth must be in contact with an antagonist in both the molar and premolar areas. Lower scores at this scale indicate more supporting zones.

Evaluation of functional capacity and depressive status

Handgrip strength in the dominant hand was measured using a handgrip dynamometer. Functional status and cognitive functions of participants from the convalescent rehabilitation hospital were assessed by the Functional Independence Measure (FIM). The FIM is the most widely accepted functional assessment measure in use in the rehabilitation community.[15] The FIM measures 18 items over six different domains: (i) self-care; (ii) sphincter control; (iii) mobility; (iv) locomotion; (v) communication; and (vi) social cognition. Thirteen items define disability in motor functions (i–iv): the FIM motor score (FIM-MS). Five items define disability in cognitive functions (v–vi): the FIM cognitive score (FIM-CS). The individual is scored on an ordered scale of 7 down to 1 on each item domain. A score of 7 is

achieved if the individual is able to perform the task independently, and 1 indicates that the individual is fully dependent on another to complete the task. The depressive symptoms were assessed by the short version of the Geriatric Depression Scale (GDS-15).[16]

Biochemical assessment

Blood samples were collected after an overnight fast. Serum total protein, albumin and total cholesterol levels were determined using automated analyzers.

Statistical analysis

Differences between two groups were determined by ANOVA or the unpaired Student's *t*-test, depending on the distribution of the analyzed variable. Results are expressed as mean ± SD. Partial rank correlation coefficients adjusted for age and sex were used to measure the relations between chewing ability (a* value) or serum albumin concentrations as a marker of nutritional status, and the other variables. To assess possible independent predictors of the chewing ability or serum albumin, stepwise multiple-regression analyses were performed. Independent variables entered into the analysis included age, sex, denture status, and those with significant correlation with chewing ability or serum albumin. All statistical analyses were performed using the SPSS statistical package software.

Results

Table 1 presents anthropometric variables, dental status such as the number of natural teeth, functional natural dentition or prostheses, and Eichner's index, chewing ability, physical and cognitive functions, depressive status, and biochemical characteristics of the participants by sex groups. The participants included 78 men (60–94 years with a mean age of 75.6 years) and 122 women (60–93 years with a mean age of 77.3 years). There were significant differences in anthropometric measurements, including bodyweight, MAC, TSF and AMA between sexes, although no differences in BMI levels were observed. With regard to biochemical variables, serum total cholesterol levels were higher in the female group. Although lower grip strength was observed in women, no differences in the number of natural teeth, Eichner's index and the chewing ability (a*)/min were detected between sexes. The proportion of those having full dentures was 20.0% (15.4% male, 23.0% female), and that of partial dentures was 33.5% (30.8% male, 35.2% female).

The correlations between the chewing ability (a*)/min and each of the variables are shown in Table 2. After

Table 1 Subject characteristics

Variable	Male		Female		*P*-value
	n	Mean ± SD	*n*	Mean ± SD	
Age (year)	78	75.6 ± 6.8	122	77.3 ± 7.3	0.098
Height (cm)	75	160.5 ± 6.0	114	147.1 ± 6.9	<0.001
Bodyweight (kg)	78	56.8 ± 11.5	122	48.1 ± 8.4	<0.001
BMI (kg/m^2)	75	22.1 ± 3.8	114	22.4 ± 3.4	0.513
MAC (cm)	73	25.0 ± 3.5	115	23.9 ± 3.7	0.043
TSF (mm)	64	12.1 ± 6.2	107	16.6 ± 8.3	<0.001
AMA (cm^2)	64	37.7 ± 11.3	105	28.8 ± 8.0	<0.001
Number of natural teeth	76	17.2 ± 11.3	114	14.3 ± 11.5	0.092
Functional natural dentition or prostheses	76	25.5 ± 6.9	114	26.2 ± 5.5	0.389
Occluding pairs of teeth (Eichner's index)	76	2.8 ± 2.4	114	2.1 ± 2.0	0.047
Chewing ability (a*)/min	78	2.6 ± 9.9	122	2.0 ± 9.9	0.658
Number of chewing strokes/min	78	74.5 ± 17.0	122	68.9 ± 15.3	0.018
Grip strength (kg)	73	25.1 ± 8.3	120	15.7 ± 5.7	<0.001
FIM-MS (range, 13–91)	31	65.7 ± 15.6	51	61.3 ± 20.2	0.272
FIM-CS (range, 5–35)	31	28.2 ± 5.9	50	27.9 ± 8.5	0.829
GDS-15 (range: 0–15)	41	6.8 ± 3.4	56	6.0 ± 3.4	0.280
Total protein (g/dL)	76	7.0 ± 0.6	118	7.0 ± 0.6	0.889
Albumin (g/dL)	74	3.9 ± 0.5	118	3.9 ± 0.5	0.835
Total cholesterol (mg/dL)	75	187.2 ± 34.7	117	208.5 ± 39.8	<0.001

BMI, body mass index; MAC, mid-upper-arm circumference; TSF, triceps skinfold; AMA, upper arm muscle area; FIM-MS, Functional Independence Measure motor score; FIM-CS, FIM cognitive score; GDS-15, the short version of the Geriatric Depression Scale.

adjusting for age and sex, there were correlations between chewing ability (a*)/min and bodyweight, MAC, the number of natural teeth, functional natural dentition or prostheses, Eichner's index, grip strength, FIM-MS and FIM-CS, GDS-15 score, serum total protein and albumin concentrations. Table 3 shows the average values of 18 variables according to chewing ability (a* value) less than 2.80 (low) and 2.80 or more

Table 2 Correlation between chewing ability (a*)/min and variables

Variable	*n*	*r*	*P*-value
Height (cm)	189	0.065	0.374
Bodyweight (kg)	200	0.173	0.015
BMI (kg/m^2)	189	0.131	0.075
MAC (cm)	188	0.183	0.012
TSF (mm)	171	0.108	0.162
AMA (cm^2)	169	0.111	0.152
Number of natural teeth	190	0.518	<0.001
Functional natural dentition or prostheses	190	0.186	0.011
Occluding pairs of teeth (Eichner's index)	190	−0.231	0.001
Number of chewing strokes/min	200	0.549	<0.001
Grip strength (kg)	193	0.251	<0.001
FIM-MS (range, 13–91)	82	0.304	0.006
FIM-CS (range, 5–35)	81	0.273	0.015
GDS-15 (range, 0–15)	97	−0.252	0.014
Total protein (g/dL)	194	0.155	0.032
Albumin (g/dL)	192	0.242	0.001
Total cholesterol (mg/dL)	192	0.115	0.114

The data were adjusted for age and sex. BMI, body mass index; MAC, mid-upper-arm circumference; TSF, triceps skinfold; AMA, upper arm muscle area; FIM-MS, Functional Independence Measure motor score; FIM-CS, FIM cognitive score; GDS-15, the short version of the Geriatric Depression Scale.

Table 3 Average values of 18 variables by chewing ability (a* value) <2.80 (low) and ≥2.80 (high)

Variable	Low		High		*P*-value
	n	Mean ± SD	*n*	Mean ± SD	
Age (year)	100	79.6 ± 6.7	100	73.6 ± 6.3	<0.001
Height (cm)	91	151.0 ± 10.1	98	153.7 ± 8.3	0.047
Bodyweight (kg)	100	49.1 ± 9.2	100	53.9 ± 11.3	0.001
BMI (kg/m^2)	91	21.7 ± 3.3	98	22.8 ± 3.7	0.045
MAC (cm)	90	23.2 ± 4.0	98	25.5 ± 2.9	<0.001
TSF (mm)	83	14.1 ± 7.7	88	15.6 ± 8.0	0.190
AMA (cm^2)	81	29.7 ± 10.1	88	34.5 ± 10.0	0.002
Number of natural teeth	94	8.5 ± 9.4	96	22.4 ± 8.8	<0.001
Functional natural dentition or prostheses	94	24.9 ± 7.6	96	27.0 ± 4.0	0.019
Occluding pairs of teeth (Eichner's index)	94	2.8 ± 2.6	96	2.0 ± 1.6	0.016
Number of chewing strokes/min	100	63.3 ± 16.2	100	78.9 ± 11.7	<0.001
Grip strength (kg)	96	16.6 ± 7.8	97	21.8 ± 7.7	<0.001
FIM-MS (range, 13–91)	44	57.6 ± 19.4	38	69.1 ± 15.7	0.004
FIM-CS (range, 5–35)	44	26.0 ± 8.1	37	30.4 ± 6.2	0.007
GDS-15 (range, 0–15)	52	7.0 ± 3.1	45	5.6 ± 3.7	0.037
Total protein (g/dL)	94	6.9 ± 0.7	100	7.1 ± 0.5	0.060
Albumin (g/dL)	92	3.8 ± 0.5	100	4.0 ± 0.4	0.001
Total cholesterol (mg/dL)	93	193.2 ± 39.4	99	206.7 ± 38.0	0.017

BMI, body mass index; MAC, mid-upper-arm circumference; TSF, triceps skinfold; AMA, upper arm muscle area; FIM-MS, Functional Independence Measure motor score; FIM-CS, FIM cognitive score; GDS-15, the short version of the Geriatric Depression Scale.