antigen receptors specific for pathogens. Activated antigen-specific T cells secrete various arrays of cytokines necessary for antibody production, and pathogen-specific antibodies play an important role in the exclusion of pathogens invading the airway, intestine and urinary tract (107–109). IgA secreted in the intestinal mucosa can neutralize toxins produced by pathogens and prevents diarrhea (110), and IgG circulating in sera is principally for defense against infection in the upper respiratory tract (107).

The incidence of infection increases and the aggravation of infectious diseases occurs when innate and acquired immune functions decline or are insufficient. Patients with undetectable levels of NK cell activity suffer frequent viral infections and the transfer of NK cells into suckling mice can render the recipient mice resistant to infection for murine cytomegalovirus (111,112). Patients with Gaucher disease, who were highly susceptible to serious bacterial infections, had macrophages with impaired anti-bacterial activity and the rate of infection among marrow transplant recipients 100–365 days after transplantation was negatively correlated with the total number of B cells and monocytes (113,114). On the other hand, several reports have shown that the improvement of depressed immune functions by ingesting foods reduced infection rates and mitigated the severity of infectious disease (43,115–118). When assessing the anti-infectious capabilities of foods, phagocytic activity, NK cell activity, T cell number, production of antigen-specific antibodies and total IgG level can be regarded as useful parameters.

NK cells exhibit cytotoxic activity against not only infected cells but also cancer cells (119–121). IFN-γ produced by activated NK cells suppresses the proliferation of cancer cells and activates cytotoxic T cells and macrophages (122,123). While NK cells kill cancer cells in an antigen non-specific manner, cytotoxic T cells recognize specific antigens of cancer cells for killing. Moreover, macrophages secrete molecules toxic to cancer cells and induce the apoptosis of cancer cells (124–126).

The proliferation and metastasis of cancer cells accelerate when immune functions are disturbed. It has been found that cancer patients have lower NK cell activity than healthy controls and persons with lower NK cell activity are subject to higher rates of cancer incidence, metastasis and aggravation of cancer (127–131). The macrophages infiltrating solid tumor have less phagocytic activity (132). On the other hand, when cancer patients ingest foods capable of improving immune functions, the prognosis becomes much better (133–137). Based on the reports of clinical trials with cancer patients, phagocytic activity, NK cell number, T cell number, DTH and IFN-γ production are all useful immune parameters for assessing the effect of foods on prognosis after surgical operation for cancer (Table 1). Moreover, it has been reported that NK cell activity deteriorates in AIDS patients (138), and branched chain amino acids, probiotics and vitamin A improves virus-triggered diseases (139–141).

Newborns exhibit immature immune functions and are vulnerable to pathogenic infection. Supplementation of vitamins in malnourished children and ingestion of probiotics in newborns enhance immune functions and prevent viral infection (118,142).

Mechanisms by which Foods Influence Immune Functions

Food-derived substances incorporated into the body via various routes modulate immune functions. Taking into consideration that malnutrition or calorie restriction cause reduced activity in immune functions, nutritional condition is indispensable for the development of the immune system (143). Moreover, food-derived substances exhibit a special role in influencing immune functions.

The way that food-derived substances modulate immune functions is either indirect or direct. Comparative analyses of conventional and germ-free animals revealed that indigenous intestinal microflora play a pivotal role in the development of host immune systems. Ingestion of probiotics stabilizes the intestinal microflora, and normalization of the intestinal microflora by probiotics could lead to modulation of the host immune system (144). In addition, probiotics such as lactic acid bacteria are recognized by specific receptors on the surface of phagocytic cells. Additionally, vitamins, minerals or fatty acids affect cellular functions by preserving the cell membrane or regulating gene expression after being incorporated into lymphocytes (Fig. 3). One group of foods represented by lactic acid bacteria stimulates innate immunity (phagocytic activity, NK cell activity), while other foods, including vitamins and minerals, activate acquired immunity (T cell response, antibody production). However, as innate immunity and acquired immunity are closely linked, both groups of foods may regulate both immune systems. It has been reported that various nutrients found in foods exhibit anti-infectious functions (145). We would like to briefly

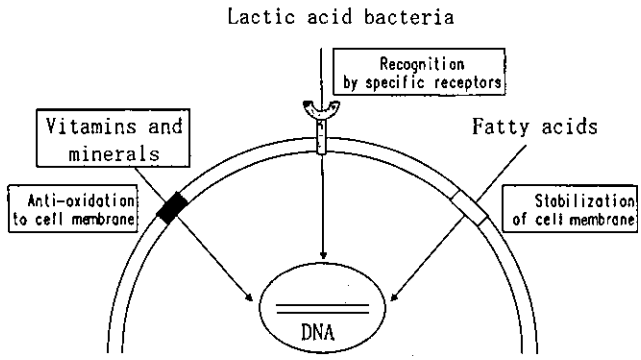

Figure 3. Mechanisms by which food-derived substances modulate the immune functions of cells. Components of probiotics are recognized by cell surface receptors such as the mannose receptor and Toll-like receptors (TLRs). It has been clarified that agonists of TLRs transmit signals to DNA by activating intracellular adaptor molecules such as MyD88. In contrast, vitamins and minerals prevent the oxidation of cell membranes and affect gene expression. Furthermore, fatty acids modulate immune functions by stabilizing the cell membrane and regulate the transcription of genes.

describe the pathways by which food-derived substances exert their immune-modulating abilities.

Probiotics ingested may be partially digested in the gut and incorporated into M cells present in FAE, and then captured by dendritic cells or macrophages in the interfollicular area of PPs. These professional phagocytic cells hold various receptors on their surface capable of binding common structures of microbes, the pathogen-associated molecular patterns (PAMPs). Among the receptors for PAMPs, molecular structure and functions of TLRs (Toll-like receptors) have been recently unveiled. Ten TLR families (TLR1–TLR10) have been identified and ligands recognized by some TLRs have been determined. TLR2 recognizes peptidoglycans and lipopeptides as TLR4 does lipoteichoic acids and lipopolysaccharides. Moreover, the CpG oligonucleotides universally detected in bacterial DNA are recognized by TLR9. The signaling response to stimuli recognized by TLRs is mainly mediated by an intracellular adaptor molecule, MyD88 (myeloid differentiation factor 88). Thereafter, the nuclear transport of NF-κB (nuclear factor-κB) is stimulated and *de novo* synthesis of cytokines is induced (146). It has been proposed that stimuli through TLR2 activate both JNK (c-Jun N-terminal kinase) and ERK (extracellular signal regulated kinase) and induce production of IL-10, while stimuli through TLR4 activate JNK and induce production of IL-12 (147).

Immune-modulating effects of amino acids such as glutamine and arginine have been evaluated. Ingestion of glutamine improved nitrogen retention and lowered incidence of bacteremia in patients with trauma, and enteral supplementation of glutamine-enriched diet enhanced the recovery of immune functions and reduced the length of hospital stay after surgical operation in cancer patients (148,149). Glutamine is a nutrient for immune cells and acts as precursor for glutathione, which circumvents oxidant stress and improves cell-mediated immunity. Arginine is a substrate for synthesis of nitric oxide and improves helper T-cell numbers. Peri-operative feeding of arginine and n-3 polyunsaturated fatty acids (PUFAs) restored DTH and decreased infection rates in colorectal cancer patients (150).

Nucleotides are rich in foods containing nucleic acid/nucleoprotein and supplementation of nucleotides is important for growth of infants. Addition of nucleotides increased the proportion of TCRγδ-bearing IELs through stimulating IL-7 production by IECs in mice (151), and ingestion of formula supplemented with nucleotides augmented NK cell activity and IL-2 production in human infants (152). It remains to be elucidated whether immune cells may utilize ingested nucleotides as substrates for synthesis of nucleic acids.

Vitamins and minerals exhibit important immune-modulating functions by entering cells and regulating gene expression. Vitamin A affects the differentiation of epithelial cells and inhibits IFN-γ production by T cells at the transcriptional level, which results in stimulation of antibody-mediated immune responses (153). Vitamin C prevents the production of reactive oxygen intermediates and reduces DNA damage in immune cells. Moreover, vitamin C inhibits the transcription of NF-κB, and down-regulates the production of pro-inflammatory cytokines (154). Vitamin E is also an anti-oxidant and exerts an anti-inflammatory effect. Vitamin E stabilizes the membrane of immune cells and enhances the binding of antigen-presenting cells and T cells (155).

Table 2. Major food-derived substances that modulate immune functions

Nutrients/nutricines	Immune-modulating functions
Nutrients/calorie	Indispensable for normal development of immune system
Amino acids	
Glutamine	Trophic for immune cells, circumvention of oxidant stress
Arginine	Substrate for synthesis of nitric oxide, enhancement of Th cells
Fatty acids	
n-3 PUFAs	Anti-inflammatory
Vitamins	
Vitamin A	Regulation of Th1/Th2 balance
Vitamin C	Circumvention of oxidant stress
Vitamin E	Circumvention of oxidant stress, anti-inflammatory
Minerals	
Selenium	Stimulation of cell-mediated immune response
Zinc	Stimulation of cell-mediated immune response
Nucleotides	Stimulation of cell-mediated immune response
Probiotics	
Peptidoglycan, lipoteichoic acids	Stimulation of IL-12/IL-10 production
CpG oligonucleotides	Anti-inflammatory

Minerals prevent the oxidation of lipids in the cell membrane, which can reduce oxidative stress affecting immune cells. For instance, selenium is indispensable to the function of reducing enzymes such as glutathione peroxidase and thioredoxin reductase, and is needed to stimulate cell-mediated immune functions (156). Furthermore, zinc may be required for the translocation and binding of NF-κB to DNA (157).

Long-chain PUFAs in foods can modulate immune functions. Dietary n-3 PUFAs alter the lipid composition of the cell membrane and regulate the function of immune cells. Antigen-presenting cells from mice and humans fed n-3 PUFAs exhibited the capacity to suppress excessive activation of T cells (158,159). As a result, n-3 PUFAs can act as anti-inflammatory agents.

Major food-derived substances and their immune-modulating functions are summarized in Table 2.

CONCLUDING REMARKS

We have reviewed and systematized studies reporting the effects of food-derived materials on immune functions in intervention studies or randomized controlled trials in order to clarify whether the immune-modulating activities of foods have been evaluated in a scientific manner. This search has revealed the following points: (i) many foods or food-derived materials improve or enhance immune functions in a wide range of human subjects; and (ii) foods with immune-modulating activities affect either innate or acquired immunity. Phagocytic activity

and NK cell activity are representative parameters of innate immunity, and phagocytes and NK cells rapidly kill pathogenic bacteria, viruses and cancer cells in an antigen-independent manner. In contrast, DTH, antigen-specific antibody production and the proliferative response of T cells are major parameters reflecting acquired immunity, which is responsible for the antigen-specific exclusion of pathogenic bacteria, viruses and cancer cells. Many kinds of foods can improve parameters exhibiting either innate or acquired immunity.

Ingestion of foods does not always change many immune parameters. Therefore, it is useful to define immune parameters affected by foods. Vitamins, minerals, amino acids, proteins, carbohydrates or lipids, for example, enhance parameters of acquired immunity. In contrast, probiotics, including lactic acid bacteria, mainly augment parameters of innate immunity. These findings support that food-derived materials act on different immune cells or distinct molecules of the cells and improve at least one parameter of either innate or acquired immunity. In other words, these results mean that one can evaluate the immune-modulating abilities of foods by analyzing parameters of either innate or acquired immunity.

The components in foods that improve immune functions and the mechanisms by which foods exert immune-modulating effects are still far from fully understood. To confirm the scientific basis of the immune-modulating activities of foods, there is a need to keep on systematizing newly obtained scientific data on foods.

Acknowledgements

Grateful thanks are extended to Dr Mitsuo Ikeda, Mr Tomoyasu Toyoda, Mr Michishiro Ito, Dr Kunio Ezawa, Dr Shigetaka Ishii, Dr Kazuo Yoshioka, Dr Hirofumi Koda, Dr Lekh Raj Juneja, Dr Yoshihiro Yamamoto, Dr Teruo Nakakuki, Dr Taizo Nagura, Mr Chiaki Sanbongi, Dr Takeshi Takahashi, Mr Hirotoshi Hayasawa and Dr Hiroshi Kawakami, all of whom are members of the Association of Food Immunology, for critical reading and helpful discussion of the manuscript.

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Received May 6, 2004; accepted August 31, 2004

臨床免疫，43(2)：156-161，2005

特集II 粘膜免疫をめぐる新たな進歩

特異抗原経口投与時における小腸上皮内リンパ球の遺伝子発現変化*

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Key Words：intestinal intraepithelial lymphocyte, interleukin-10, ovalbumin, TCR transgenic mouse

はじめに

腸管粘膜は常在細菌や食物由来抗原に常に曝されている．これら管腔内抗原との相互作用の最前線にある腸管上皮細胞の間に存在するT細胞が，腸管上皮内リンパ球(intestinal intraepithelial lymphocyte；IEL)である[1)2)]．IELはその数においてIEL以外の通常のリンパ組織に存在するT細胞に匹敵するが，これらとは性状が異なる独自の細胞群である．IELはT細胞レセプター(TCRαβ, TCRγδ)および補助レセプター分子(CD4, CD8αα, CD8αβ)の発現により異なるサブセットに分類され，それぞれ異なる生理機能を果たすものと考えられる．IELの生理機能としては，上皮層の恒常性維持や感染防御，免疫応答の制御などが報告されているが，いまだ十分には明らかにされていない．

$CD8\alpha\alpha^+$ $TCR\alpha\beta^+$IEL(αβ-IEL)と$TCR\gamma\delta^+$IEL(γδ-IEL)については，胸腺外分化IELであり，粘膜固有層に存在するクリプトパッチに由来するものであることが報告された[3)4)]が，$CD8\alpha\alpha^+$ αβ-IELは胸腺の$CD4^+CD8^+$細胞に由来するという説[5)6)]もあり，いまだ結論は得られていない．これらのIELは自己抗原や非古典的MHCクラスIbなどを認識するものと考えられるが，その詳細は不明である．

一方，通常の末梢リンパ組織にも存在する$CD4^+$あるいは$CD8\alpha\beta^+$の表現型をもつαβ-IELと$CD4^+$ $CD8\alpha\alpha^+$ αβ-IEL[7)]は，おそらく末梢で活性化されたT細胞が上皮内にホーミングしてIELとなったものであり，管腔内の抗原に対する特異的な反応を担っているものと考えられる．しかしながら，その生理機能，とくに経口摂取された食物由来抗原に対するIELの応答や生理的意義についてはいまだ不明な点が多い．

われわれは，IELが食物抗原の認識にかかわる可能性およびそこでIELが果たす役割について検討するため，MHCクラスII分子拘束的に卵白アルブミン(OVA)を認識するTCRトランスジェニックマウスを用い，OVAを経口的に摂取させた場合にIELに生じる変化を，とくに遺伝子発現変化に注目して解析した．

特異抗原の経口摂取によりIELに生じる変化

OVA-TCRトランスジェニックマウス(DO11.10マウス)にOVAを含む食餌(卵白食)あるいは，カゼインを含む食餌(カゼイン食：対照食)を自由摂取させ，特異抗原の経口摂取によりIELに生じる変化について検討を行った．フローサイトメ

* Altered gene expression in intestinal intraepithelial lymphocytes derived from mice fed a specific antigen.

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トリーによりIELサブセット構成を解析した結果，3日間の卵白食摂取によってCD4⁺CD8αα⁻ αβ-IELが増加することが示された．

また，3日間の卵白食摂取により，DO11.10マウスTCRのクロノタイプ抗体であるKJ1.26⁺のIELにおいてCD69の発現上昇，CD45RBの発現低下，CD62L⁺細胞の増加が明らかとなった．CD69の発現上昇はCD4⁺IELおよびCD8α⁺IELで，CD62L⁺細胞の増加はCD8α⁺IELで認められた．一般に末梢T細胞においてCD69は初期活性化マーカーであり，一方CD62Lは活性化に伴い発現低下することが知られている分子であり，この結果が意味するところを明らかにするには，さらに詳細な解析が必要であろう．しかしながら，これらの結果から，特異抗原を経口摂取することにより，IELにおいて少なくともなんらかの変化が生じることが明らかとなった．この結果の解釈にあたっては，①IELが抗原提示を受け活性化した，あるいは②ほかのリンパ球が抗原を認識しIELに作用を及ぼした，という両者が考えられる．

CD4⁺ IELが管腔内抗原に対して反応することに関しては，これまでにも報告がある[8][9]．一方，食物抗原の摂取に対してIELが反応する例としては，セリアック病患者において，グルテンの経口摂取がIELを増加させることが知られている[10]．またわれわれのグループは，DO11.10マウスのTCRと同じ抗原を認識するOVA-TCRトランスジェニック(OVA23-3マウス)を用い，同様にOVAを経口摂取させることにより，CD4⁺CD8αα⁻ αβ-IELが増加し，*in vitro*抗原刺激に対する増殖応答性が高まることを報告している[11]．これらの事実から，IELが食物抗原に対してもなんらかの反応を示すことは確かであると考えてよいであろう．

特異抗原の経口摂取によるIELの遺伝子発現変化

次に特異抗原の経口摂取でIELに生じる変化を分子レベルで明らかにするため，DNAマイクロアレイ法により遺伝子発現変化を解析した．卵白食あるいは対照食を3日間自由摂取させた群から，それぞれIELを調製し，セルソーターによりTCRαβあるいはTCRγδを発現するIEL(whole IELとする)を精製した．GeneChip®システム(Affymetrix社)を用いて両群のwhole IELの遺伝子発現の違いを検討した結果，2回の実験でともに卵白食摂取によりIELにおいて有意に発現が上昇した遺伝子98個，発現が低下した遺伝子24個を同定した(表1)．発現が上昇した機能分類別の遺伝子数としては，半数近くが細胞周期・細胞増殖に関する遺伝子であり，次いで，免疫・炎症関連，細胞骨格関連，転写調節関連の遺伝子が多く認められた．2回の実験でともに3倍以上の発現増加が認められたものを表2に示した．また，CD4遺伝子の発現上昇が検出されており，これはCD4⁺ CD8αα⁻ αβ-IELが増加したことと符合する結果である．

表1 特異抗原の経口摂取によりwhole IELにおいて発現の変化した遺伝子の機能別分類

機能別分類	発現上昇（遺伝子数）	発現低下（遺伝子数）
細胞周期・細胞増殖	44	1
免疫関連	14	2
細胞骨格	12	0
転写因子・転写調節	8	1
代謝・ハウスキーピング	8	6
シグナル伝達	3	1
アポトーシス	3	0
そのほか・機能未知	2	6
ESTs	4	7
合計	98	24

DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，TCRαβ⁺あるいはTCRγδ⁺IEL(whole IEL)をセルソーターにより精製した．whole IELからRNAを抽出し，Affymetrix社のGeneChip®システム(Murine Genome U74Av.2 Array)により遺伝子発現の解析を行った．2回の実験でともに，対照食群と比較して卵白食群で発現が上昇あるいは低下した遺伝子数を示した．

細胞周期・細胞増殖に関する遺伝子の発現増大が顕著に認められたことから，IEL中に食物抗原を認識して増殖した細胞が含まれていることが推測された．一般に，IELは*in vitro*で抗原刺激をした場合，その増殖性は低いことが知られている．IELの中で増殖関連遺伝子の発現が上昇する細胞は，おそらくOVAを認識するKJ1.26⁺細胞であることが予想され，ここでも次の2つの可能性が考えられる．すなわち，①IELが上皮内で抗原を認識し増殖した，②パイエル板，腸間膜

表2　特異抗原の経口摂取によりwhole IELにおいて発現が上昇した遺伝子

Accession No.	遺伝子名	発現倍率（卵白食群/対照食群）	
		実験1	実験2
細胞周期・細胞増殖			
AF002823	budding uninhibited by benzimidazoles 1 homolog (Bub1)	16.0	26.0
AF016583	checkpoint kinase 1 homolog (Chek1)	11.3	3.5
AW213883	RIKEN cDNA A730011O11 gene (A730011O11Rik)	9.9	4.9
X60980	thymidine kinase 1 (Tk1)	8.0	4.9
AW209238	transforming, acidic coiled-coil constaning protein 3 (Tacc3)	5.7	19.7
AB025409	CDC28 protein kinase 1 (Cks1)	4.9	26.0
MI4223	ribonucleotide reductase M2 (Rrm2)	4.9	12.1
U01915	topoisomerase (DNA) II alpha (Top2a)	3.7	42.2
X66449	S100 calcium binding protein A6 (S100a6,calcyclin)	3.7	4.0
AJ223087	cell division cycle 6 homolog (Cdc6)	3.5	7.0
AI317217	cyclin-dependent kinase inhibitor 3 (Cdkn3)	3.5	9.8
X82786	antigen identified by monoclonal antibody Ki 67 (Mki67)	3.3	9.8
免疫関連			
M37897	interleukin 10 (IL-10)	9.2	22.6
X15986	lectin, galactose binding, soluble (Lgals1, galectin1)	3.3	8.0
細胞骨格			
AJ223293	kinesin family member 11 (Kif11)	4.9	13.0
AI591702	kinesin family member 23 (Kif23)	3.5	4.3
転写因子・転写調節			
M90397	B-cell leukemia/lymphoma 3 (Bcl3)	9.2	4.0
代謝・ハウスキーピング			
D55720	karyopherin (importinn) alpha 2 (Kpna2)	-3.3	3.7
シグナル伝達			
AI838080	stathmin 1 (Stmn1)	4.6	21.1
EST			
AI122538	RIKEN cDNA 2810417H13 gene (2810417H13Rik)	4.9	21.1

表1と同じ実験において，2回の実験でともに3倍以上，対照食群と比較して卵白食群で発現が上昇した遺伝子を示した．

リンパ節などで抗原を認識し増殖過程にあるT細胞が上皮内に移動した，という可能性である．

IELにおけるIL-10遺伝子発現およびIL-10産生の上昇

免疫関連遺伝子のうち，特異抗原の経口摂取でもっとも顕著な発現上昇が認められたのはIL-10遺伝子であった．定量的リアルタイムRT-PCR法により，IL-10遺伝子の発現を確認したところ，卵白食摂取期間が3日，7日，14日のいずれにおいても，whole IELにおいて，対照食群と比較してIL-10遺伝子の発現上昇が認められた．また，両群から分離したwhole IELを抗CD3抗体および抗CD28抗体を用いて*in vitro*で刺激した場合の培養上清中へのIL-10分泌を測定したところ，蛋白質レベルでも卵白食摂取群のIELでより強いIL-10発現が確認された(図1)．一方，ほかのサイトカイン遺伝子については，DNAマイクロアレイ解析からは卵白食摂取で増加するものは認められなかった．IL-4およびIFN-γについては，RT-PCRおよび*in vitro*抗原刺激によるサイトカイン産生試験でも検討したが，ともに両群で差が認められなかった(図1)．

IL-10遺伝子を発現するIELサブセット

次にwhole IELのうち，どの画分がIL-10遺伝子を発現しているのかを検討した．BALB/cマウスより調製したIELを$CD4^+$IELと$CD4^-$IELに分画し，IL-10遺伝子発現を調べたところ，$CD4^+$IELにおいて顕著な遺伝子発現が検出された(図2)．また，ほかのリンパ組織についても検討したところ，パイエル板$CD4^+$T細胞で発現が認められ

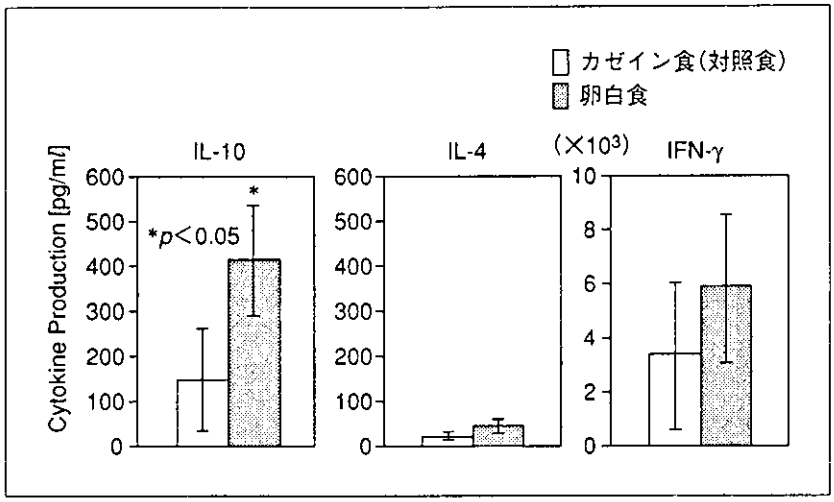

図1　特異抗原の経口摂取によるwhole IELのサイトカイン産生の変化
DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，TCRαβ⁺あるいはTCRγδ⁺IELをセルソーターにより精製した．これを抗CD3抗体および抗CD28抗体で刺激した後，培養上清中のサイトカイン産生量をELISA法で測定した．

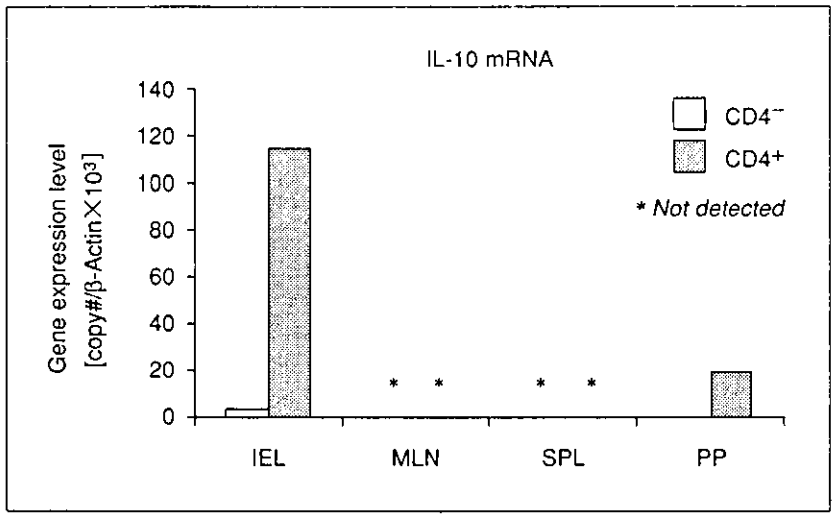

図2　IEL，腸間膜リンパ節(MLN)，脾臓(SPL)およびパイエル板(PP)由来CD4⁺あるいはCD4⁻T細胞におけるIL-10遺伝子発現の比較
BALB/cマウスの各リンパ組織よりT細胞を調製し，CD4⁺およびCD4⁻画分に分画した．各細胞よりRNAを抽出し，リアルタイムPCRを用いた定量的RT-PCR法によりIL-10遺伝子の発現を測定した．

たが，CD4⁺IELと比べるとその発現は低かった．腸間膜リンパ節，脾臓においては，CD4⁺T細胞およびCD4⁻T細胞ともにIL-10遺伝子の発現は認められなかった．この実験は，*ex vivo*で生体から取り出したままの細胞からRNAを抽出しIL-10 mRNAの存在量を解析したものであり，*in vitro*でのTCR刺激を加えていない状態の結果であることに注意が必要である．また，この結果は通常のマウスを用いて得られたものであり，IELに対して*in vivo*においても人為的な抗原刺激は行っていないことも重要なポイントである．したがって，CD4⁺IELは①管腔内抗原の刺激により，あるいは②上皮細胞間という環境下にあることにより，恒常的にIL-10遺伝子を発現していることが示された．どちらの影響によるものであるかについては今後の検討が必要である．

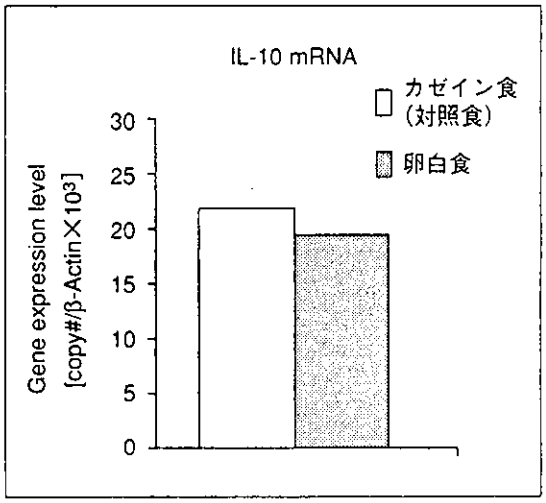

図3　卵白食あるいは対照食を摂取したDO11.10マウス由来のCD4⁺IELにおけるIL-10遺伝子の発現
DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，CD4⁺IELをセルソーターにより精製した．細胞からRNAを抽出し，リアルタイムPCRを用いた定量的RT-PCR法によりIL-10遺伝子の発現を測定した．

一方，卵白食あるいは対照食を摂取させたDO11.10マウスからCD4⁺IELを精製してIL-10遺伝子の発現を調べたところ，両者の間には顕著な差が認められなかった(図3)．すなわち，特異抗原の経口摂取によるIELのIL-10遺伝子発現増強に対して，CD4⁺IEL1細胞あたりのIL-10遺伝子発現量の上昇が寄与するところは少なく，主にIL-10遺伝子を発現するCD4⁺IELの数の増加によって生じたものであることが示唆された．また，腸間膜リンパ節CD4⁺T細胞を*in vitro*抗原刺激した場合，対照食群ではIL-10産生は認められなかったが，卵白食摂取でその産生が認められた．しかしながら，1細胞あたりのIL-10産生量は，腸間膜リンパ節CD4⁺T細胞よりも，CD4⁺IELの方が高いことが示された．

ある種の制御性T細胞(Tr1)はIL-10を高産生し，その分化発達にはIL-10が必要であることから[12]，CD4⁺IELが食物抗原に対する免疫応答や炎症反応の抑制的制御に関与していることが推察された．SCIDマウスに脾臓CD4⁺CD45RB^{hi}T細胞を移入して起こる腸炎症は，CD8αα⁺ αβ-IELを移入することにより抑制されることが報告されている[13]．この抑制はIL-10$^{-/-}$マウス由来の同細胞ではみられないことから，この反応がIL-10依存的であることは明らかにされているが，この細胞を抗CD3抗体および抗CD28抗体で刺激してもIL-10を産生しない[13]．また，αβ-IELにおけるIL-10遺伝子発現は低いという結果も得られているが[14]，αβ-IEL中のCD4⁺IELの存在比が小さいことを考えると理解できる結果である．われわれの結果を合わせて考えると，CD4⁺IELがIELにおけるIL-10の主な供給源となっていることが示唆される．DasらはCD4⁺CD8αα⁺ αβ-IELがIL-10を産生し，CD4⁺CD45RB^{hi}T細胞の移入で起こる腸炎症をIL-10依存的に抑制することを報告している[7]．われわれの結果では，特異抗原の経口摂取によりCD4⁺CD8αα⁻ αβ-IELが増加したことから，このIELサブセットがIL-10遺伝子発現増大に寄与しているものと考えられる．したがって，IL-10遺伝子を高発現するCD4⁺CD8αα⁻ αβ-IELもCD4⁺CD8αα⁺ αβ-IELと同様に制御性T細胞としての機能を有している可能性が考えられる．また，食物抗原の摂取によりこのような制御性T細胞がIEL中に誘導されてくる可能性が示唆された．

おわりに

本研究ではDNAマイクロアレイ解析により，特異抗原の経口摂取によって，IELに増殖中の細胞が増加すること，小腸上皮内にCD4⁺CD8αα⁻ IELが増加すること，CD4⁺IELはIL-10遺伝子を恒常的に発現していることが示された．これらの結果から示唆された事象については，今後実験的な裏づけをしていく必要がある．これまでに主にγδ-IELとαβ-IELの異同に着目し，網羅的な遺伝子発現解析により特徴的な発現を示す分子からIELの特性・機能を推定する研究が報告されている[14)~16)]．各IELサブセットでの発現が明らかにされた分子に注目して研究を進めることで，IELの分化経路や生理機能などに新しい知見が得られることが期待される．

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J Clin Periodontol 2005; 32: 93–97, doi: 10.1111/j.1600-051X.2004.00643.x

Journal of Clinical Periodontology

Serum vitamin C–periodontal relationship in community-dwelling elderly Japanese

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Amarasena N, Ogawa H, Yoshihara A, Hanada N, Miyazaki H: Serum vitamin C-periodontal relationship in community-dwelling elderly Japanese. J Clin Periodontol 2005; 32: 93–97. doi: 10.1111/j.1600-051X.2004.00643.x. © Blackwell Munksgaard, 2004.

Abstract

Objective: To determine the relationship between serum vitamin C and periodontitis as estimated by clinical attachment loss (CAL) in community-dwelling elderly Japanese.

Material and Methods: This analysis was confined to 413 Niigata citizens aged 70 years in whom the data for serum vitamin C and CAL were available. High-pressure liquid chromatography method was used to ascertain the serum vitamin C levels while CAL was assessed on six sites of all teeth present including third molars by means of pressure-sensitive probes. Other variables included gender, smoking, toothbrushing frequency, number of teeth present and random blood sugar levels.

Results: Serum vitamin C concentration was inversely related to CAL ($r = -0.23$, $p < 0.00005$) at bivariate level. Multiple linear regression analysis showed that CAL was 4% greater in subjects with lower serum vitamin C levels than in subjects with higher serum vitamin C levels notwithstanding smoking, diabetes, oral hygiene, gender or number of teeth present.

Conclusion: The findings suggested that serum vitamin C might have relatively weak but a statistically significant relationship with periodontitis in this elderly population.

Key words: elderly; periodontitis; serum vitamin C

Accepted for publication 27 April 2004

Investigations into vitamin C (ascorbate/ascorbic acid)–periodontal relationship go as far back as the 18th century when a British naval physician revealed that scurvy, which was accompanied by putrid gums could be successfully treated with oranges and lemons (Rubinoff et al. 1989). Since then numerous experimental as well as epidemiological studies in both humans and animals have attempted to address this issue but the findings have been rather incoherent: some have failed to suggest any significant relationship between vitamin C and periodontal disease (Waerhaug 1958, Barros & Witkop 1963, Russel et al. 1965, Enwonwu & Edozien 1970, Woolfe et al. 1980, 1984) while others reported that the deficiency of this vitamin could not be correlated with severe periodontitis but with gingival inflammation or acute necrotizing ulcerative gingivitis (Enwonwu 1972, Shannon 1973, Leggott et al. 1986, Melnick et al. 1988). Still others have observed a weak association between vitamin C and periodontitis (Ismail et al. 1983, Nishida et al. 2000). However, the majority of workers who have looked into ascorbic acid–periodontal relationships in humans estimated the dietary intake of vitamin C (Ismail et al. 1983, Nishida et al. 2000) in comparison to the few who have assessed serum or plasma ascorbic acid levels (Leggott et al. 1986, Melnick et al. 1988, Pussinen et al. 2003), which might provide much reliable information than the former method (Simon & Hudes 2001). On the other hand, little or virtually nothing has been reported in the literature on the association between vitamin C and periodontal status of the elderly who may be at a higher risk of developing vitamin C deficiency compared with other age groups (Rubinoff et al. 1989, Pussinen et al. 2003). In view of these facts, it is important to explore the link between serum ascorbic acid levels and periodontitis in an elderly population. Accordingly, the main purpose of the present investigation was to ascertain the relationship between serum vitamin C levels and periodontitis as measured by clinical attachment loss (CAL) among senior citizens in Japan.

Material and Methods

This was a part of the ongoing oral and general health survey, which has been carried out in senior citizens of Niigata city, Japan since 1998. The study methodology has been described in

detail elsewhere (Hirotomi et al. 2002, Ogawa et al. 2002). A written invitation was sent to all individuals aged 70 years ($n = 4542$; 2099 males and 2443 females) who were registered as the citizens of Niigata city, Japan in 1998 to take part in this survey once the Ethical Committee of the School of Dentistry, Niigata University, approved the research protocol of the survey. After sending a second request, 79.5% (3695) of the population consented to participate in the survey. Having considered the resources available, out of the positive respondents 600 individuals were randomly recruited into a cross-sectional community-based study so as to have an approximately equal number of males (306) and females (294). Informed consent was obtained from all subjects prior to the investigation.

Four calibrated dentists conducted the intra-oral examination involving assessment of CAL, probing depth (PD) and bleeding on probing (BOP) using mouth mirrors and pressure-sensitive TPS Probe® (Vivacare, Schaan, Liechtenstein) under artificial light. All teeth present including third molars were probed at six sites per tooth, namely, mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual and mesio-lingual and the recordings were rounded up to the nearest whole millimetre. Calibration of the examiners was carried out before and during the survey and the examiner consistency ranged from 0.56 to 0.92 as indicated by κ statistic. Information pertaining to smoking habits as well as oral hygiene practices was obtained by means of a personal interview, whereas blood samples were sent to the laboratory in order to evaluate the serum vitamin C levels using high-pressure liquid chromatography (HPLC) method and random blood sugar levels.

Statistical analyses were carried out by means of STATA statistical software package. Throughout the analysis, CAL, the dependent variable, was considered as a continuous variable and the unit of analysis was the subject. Among independent variables, serum vitamin C level was a continuous variable while gender (male:female), smoking status (current smoker: ex-smoker: non-smoker), diabetic status (random blood sugar (RBS) < 140 mg/dl: $\geqslant 140$ mg/dl), frequency of tooth cleaning (< 2/day: $\geqslant 2$/day) and the number of teeth present (< 20 teeth: $\geqslant 20$ teeth) were treated as categorical variables. To compare the difference between two means Student's *t*-test was employed while one-way ANOVA combined with Bonferroni's test was used where necessary to compare more than two means. Moreover, the association between two continuous variables was determined by means of Pearson's correlation technique. Finally, having excluded the presence of multicolinearity, the independent variables that showed significant relationships with CAL at bivariate level were included in a multiple linear regression analysis to identify the independent effect of serum vitamin C level on CAL while controlling for other confounding factors. The level of statistical significance was fixed at $p \leqslant 0.05$.

Results

The current analysis was limited to 413 dentate subjects in whom the data for CAL as well as serum concentrations of vitamin C were available. The serum levels of vitamin C ranged from 0.2 to 22.6 mg/l with a mean of 7.21 (results not shown). Table 1 shows the periodontal characteristics of the sample. Accordingly, the mean CAL was 3.26 mm (SD = 1.05) while the mean PD was 2.1 mm (SD = 0.58). The mean percentage of BOP per person was 7.3 (SD = 8.6). It was also observed that 62.6% of subjects had at least one site with CAL of $\geqslant 6$ mm, whereas almost one-third (33%) of the sample exhibited PD of $\geqslant 6$ mm at least on one site (results not shown). Indeed, there was no significant relationship between serum vitamin C levels and either PD or BOP (results not shown) and consequently, it was decided to confine the present analysis to explore the relation between the independent variables including serum vitamin C concentration and CAL. Table 2 depicts the associations between CAL and the independent variables including gender, smoking status, diabetic status, brushing frequency, the number of teeth present and serum vitamin C concentrations. As revealed by Student's *t*-test, males had significantly greater CAL (mean = 3.54 mm; SD = 1.2) than females (mean = 2.96 mm; SD = 0.8 mm) while those who had 20 or more teeth showed significantly lower CAL (mean = 2.92 mm; SD = 0.8) compared with the subjects with < 20 teeth (mean = 3.69 mm; SD = 1.2). It is also apparent that CAL was significantly higher in the

Table 1. Periodontal characteristics of the sample

Variable	Mean	SD
CAL (mm)	3.26	1.05
PD (mm)	2.10	0.58
BOP (%)	7.30	8.60

CAL, clinical attachment loss; PD, probing depth; BOP, bleeding on probing

Table 2. Relationships between CAL and independent variables at bivariate level

Independent variables	CAL Mean (SD)	*p*
Gender*		
male ($n = 215$)	3.54 (1.2)	< 0.00005
female ($n = 198$)	2.96 (0.8)	
Number of teeth present*		
< 20 ($n = 184$)	3.69 (1.2)	< 0.00005
$\geqslant 20$ ($n = 229$)	2.92 (0.8)	
Brushing frequency*		
< 2/day ($n = 145$)	3.40 (1.1)	< 0.05
$\geqslant 2$/day ($n = 268$)	3.20 (1.0)	
Smoking status†		
current smoker ($n = 71$)	3.82 (1.3)	< 0.00005‡
ex-smoker ($n = 137$)	3.45 (1.1)	
non-smoker ($n = 205$)	2.95 (0.8)	
Diabetic status*		
RBS < 140mg/dL (n = 356)	3.22 (0.1)	< 0.05
RBS $\geqslant 140$ mg/dl ($n = 57$)	3.52 (0.2)	
Serum ascorbic acid§	$r = -0.23$	< 0.00005

CAL, clinical attachment loss; RBS, random blood sugar. *Student's *t*-test.
†One-way ANOVA.
‡Bonferroni's test: $3.82 > 3.45 > 2.95$ ($p < 0.05$).
§Pearson's correlation.

Table 3. Multiple linear regression model for CAL with significant variables

Independent variables	Coefficient	SE	p	95% CI	
Serum ascorbic acid	−0.04	0.02	<0.05	−0.06	−0.005
Current smoker	0.57	0.17	<0.005	0.24	0.92
Gender (male = 0)	−0.30	0.04	<0.05	−0.58	−0.01
Teeth present (<20 = 0)	−0.73	0.09	<0.0005	−0.92	−0.55
Constant	3.83	0.25	<0.0005	3.46	4.20

$R^2 = 0.26$; $p < 0.00005$; SE, standard error; CI, confidence interval.

subjects who brushed their teeth <2/day than in those who used a toothbrush ≥2/day. One-way ANOVA combined with Bonferroni's post hoc test disclosed that current smokers had significantly worse CAL (mean = 3.82 mm; SD = 1.3) in comparison with both ex-smokers (mean = 3.45 mm; SD = 1.1) and non-smokers (mean = 2.95 mm; SD = 0.8), whereas subjects with RBS <140 mg/dl showed significantly lower CAL than those who had RBS≥ 140 mg/dl. Furthermore, there was an inverse relationship between serum vitamin C concentration and CAL as indicated by Pearson's correlation technique ($r = -0.23$; $p < 0.00005$).

All the independent variables that demonstrated significant effects on CAL at bivariate level, namely, serum vitamin C, smoking status, diabetic status, gender, toothbrushing frequency and the number of teeth present were included in a multiple linear regression analysis and the variables that remained significant in the final model are shown in Table 3. Accordingly, it was found that serum vitamin C had a significant effect on CAL (correlation coefficient = −0.04; $p < 0.05$), which was independent of the other covariates including smoking and random blood sugar levels. The independent variables in the final model explained 26% of the variance in CAL ($R^2 = 0.26$).

Discussion

The findings of this cross-sectional study suggested that there was a weak but significant association between the level of serum vitamin C and periodontitis as measured by CAL notwithstanding the effect of established risk factors for periodontitis such as smoking and diabetes mellitus in this elderly population. In other words, we observed an inverse independent relationship between serum vitamin C concentration and CAL – the lower the level of serum vitamin C the higher was the periodontal attachment loss. This was indicated by the relatively smaller correlation coefficient of serum vitamin C (correlation coefficient = −0.04): CAL in subjects with lower serum vitamin C levels would only be 4% greater compared with those who had higher serum vitamin C concentrations regardless of other covariates.

Notwithstanding the fact that our study was confined only to the elderly and that we evaluated serum ascorbic acid concentration instead of dietary intake of vitamin C, the present findings may be comparable to those of others (Ismail et al. 1983; Nishida et al. 2000) who observed a weak albeit statistically significant relationship between dietary vitamin C and periodontal disease in the US adults. In particular, the latter (Nishida et al. 2000) found that even after controlling for the effects of age, gender, smoking and gingival bleeding, the level of periodontitis in subjects with a lower dietary intake of vitamin C was 1.19 times greater than that of individuals with a higher intake of vitamin C while the former (Ismail et al. 1983) did not adjust for such factors. More recently, Pussinen et al. (2003) who investigated the relation between plasma vitamin C levels and serology of periodontitis in Finnish and Russian men observed that the antibody levels to *Porphyromonas gingivalis* were inversely correlated with plasma vitamin C concentrations ($r = -0.22$; $p < 0.001$) and this association remained significant in a linear regression model even after controlling for confounding factors. Accordingly, they concluded that lower concentrations of plasma vitamin C might increase the risk of periodontitis, which is in accord with the present findings.

Various researchers have proposed several plausible biological mechanisms while attempting to explain how ascorbic acid could affect the healthy tissues in humans as well as in animals (Goetzl et al. 1974, Alfano et al. 1975, Boxer et al. 1979, Dallegri et al. 1980, Alvares et al. 1981, Alvares and Siegel 1981, Berg et al. 1983, Leggot et al. 1986, Jacob et al. 1987, Nakamoto et al. 1984). It has been established that ascorbic acid plays a major role in the synthesis of collagen, especially the hydroxylation process, helix formation and cross-linking of collagen molecules (Alfano et al. 1975, Berg et al. 1983). Collagen is undoubtedly an essential component of human tissues including periodontium and required in wound healing as well as periodontal regeneration and maintaining the integrity of the gingival vasculature. Also, there are several lines of evidence to suggest that vitamin C affects chemotaxis as well as phagocytosis of polymorphonuclear leucocytes and thereby influences the host-immune reactions (Alfano et al. 1975, Boxer et al. 1979, Dallegri et al. 1980, Patrone et al. 1982). Moreover, some researchers have hypothesized that ascorbic acid might express an antihistamine effect through direct detoxification of histamine or indirectly affecting the histamine breakdown and this in turn would retard gingival inflammation (Nakamoto et al. 1984) whereas others (Alfano et al. 1975, Alvares and Siegel 1981) reported that the deficiency in vitamin C levels could be linked to increased permeability of gingival mucosa, which allows easy passage of microbial and other noxious products into the periodontium. It has also been shown that ascorbic acid demonstrates antioxidant properties and therefore is considered one of the constituents of antioxidant defence mechanism in human body (Nishida et al. 2000). Tobacco, especially, cigarette smoke contains various oxidants that cause tissue damage and consequently smokers do require a higher serum concentration of vitamin C than non-smokers do (Kallner et al. 1981, Nishida et al. 2000). Moreover, given that avitaminosis C and diabetes mellitus share some common pathological characteristics such as raising of oxidant stress (Schmidt et al. 1996) and collagen degradation (Kjersem et al. 1988) in gingival tissues, it has been hypothesized that vitamin C might play a critical role in the aetiology and/or progression of periodontitis in type I diabetics (Aleo 1981, Nishida et al. 2000). In this connection, it is also noteworthy that both diabetes as well as smoking, which are regarded as well-established risk factors for periodontitis, may contribute to oxidative tissue damage and given the antioxidant properties

of vitamin C, it might act as a potential moderator in both smoking– and diabetes–periodontal relationships – this would be an interesting hypothesis to be tested in future investigations. Although exploring such biological mechanisms and/or hypotheses was beyond the scope of our study, the association between serum vitamin C levels and CAL that was observed even after controlling for known risk factors such as smoking and diabetes mellitus in the present study could be explained on the basis of these mechanisms. This is further augmented by the fact that such mechanisms could be connected to pathogenesis of periodontal disease, which is of inflammatory nature and which may be mediated through the tissue damage caused by interaction of microbial noxious products and host-immune response. However, it should also be highlighted that the observed relationship is rather weak and that these biological phenomena involving vitamin C have neither been clearly understood nor well defined (Leggot et al. 1986, Nishida et al. 2000, Pussinen et al. 2003).

This study population comprised non-institutionalized elderly people who were active, living independently and willing to participate in the survey. It has been shown that the elderly who are institutionalized, less active and dependent are at a higher risk for periodontal disease than those who are active and independent (Hirotomi et al. 2002, Ogawa et al. 2002). Besides, the mean serum concentration of vitamin C in this sample was rather high and the serum vitamin C level in only about 4% of subjects (results not shown) was below the reference range for Japanese elderly (Sakai et al. 1998). In this context, the current sample might be considered a biased one and therefore the findings should be interpreted with caution.

In conclusion, the results suggest that the serum vitamin C levels in this elderly population weakly correlate with periodontitis as evaluated by CAL notwithstanding the effects of smoking, diabetes mellitus, gender, oral hygiene practices or the number of teeth present. Moreover, considering the cross-sectional nature of the study design it was almost impossible for us to ascertain lifetime changes in either the vitamin C intake or serum vitamin C levels in this population. Because of these facts and also given the relatively low correlation observed between serum vitamin C and CAL in the current analysis, we could neither confirm an unambiguous cause–effect relationship between serum vitamin C and periodontitis nor a substantial beneficial effect of vitamin C on periodontal health. All in all, the association observed here could not be a straightforward one but it would be plausible that the serum vitamin C might be inflicting a moderating influence on periodontitis through the established risk factors such as smoking and diabetes, as it was mentioned hitherto. Consequently, it warrants further investigations, in particular, longitudinal studies and experimental designs to explore the actual role of vitamin C in the aetiology and/or progression of periodontal disease. It should also be highlighted that this elderly cohort will be followed up for several years and thus, we intend to analyse the serum vitamin C–periodontal relationship prospectively, in the same population.

Acknowledgments

This investigation was supported by Grant-in-Aids from Japan Society for the Promotion of Science (01340) and from the Ministry of Health and Welfare of Japan (H10-Iryo-001).

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Journal of Oral Science, Vol. 46, No. 2, 127-133, 2004

Original

Characteristics and willingness of patients to pay for regular dental check-ups in Japan

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(Received 1 December 2003 and accepted 13 April 2004)

Abstract: The purpose of this survey was to investigate the relationship between demographic characteristics and willingness of patients to pay for regular dental check-ups in Japan. Questionnaires were distributed at private dental offices and collected via postage-paid return envelopes addressed to the center of the study groups. Questions focused on demographics and willingness to pay for regular check-ups. Five thousand one hundred thirty-two questionnaires were collected (response rate 56.8%). The 3 groups most likely to have regular dental check-ups were found to be the under 20s, 50 to 59 year olds and civil servants. Of these groups, civil servants were found to be the most likely of all to have regular check-ups. More females than males were represented in the sample. More than 60% of the patients responded that they would be willing to pay for regular check-ups if the cost were less than 2,000 yen (about $ 20). However, no statistically significant differences were observed in relation to household income. The results suggested that participation in regular dental check-ups might be related to gender and age, but not to household income. (J. Oral Sci. 46, 127-133, 2004)

Key words: regular check-ups; willingness to pay; household income.

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Introduction

The decline of the prevalence of dental caries is a worldwide trend (1). This tendency has also been observed in Japan. The national survey for oral diseases, carried out every six years, indicates a tendency toward the decline of dental caries in the deciduous teeth and permanent teeth of the young adult population (2). The services provided by dental professionals have therefore gradually changed from the treatment of dental caries to the provision of regular check-ups and professional preventive programs. However, an increase in periodontal disease has nevertheless been observed (3,4). In Japan, for example, according to a survey in 1999, 72.9% of subjects aged 5 years and older had periodontal disease (2). Prevalence was also found to increase with age, with 88.4% of subjects in the 45- to 54-year-old range having periodontal disease (2). Another survey showed that only 54.4% of patients visit clinics to treat periodontal disease (5). Consequently, there is still a need for regular check-ups and dental care to prevent dental caries (6,7). Furthermore, the need to prevent and treat periodontal disease is high. However, these needs are not always attended to at the dental clinic visited.

The effect of regular check-ups on the prevention of dental caries has not been clearly established. It has been shown that decreasing the frequency of regular check-ups does not lead to an increase in dental caries (8). Another report showed that the incentive of a capitation system reduced dental caries, and the number of deep periodontal and bleeding pockets (9,10). This difference may be derived from the extent of dental care provided at

regular check-ups. In Japan, regular check-ups for post-adolescents are not provided by the public health service. Subjects must therefore obtain these services at private dental clinics, even though Japanese health insurance does not cover the check-ups. The national average percentage of regular check-ups per clinic was less than 5% (7); a figure we consider to be less than satisfactory.

The purpose of this survey was to investigate the relationship between demographic characteristics and willingness of patients to pay for regular dental check-ups in Japan. The goals of our study were to investigate and improve the skills of dentists and dental hygienists working in private dental clinics to better prevent oral diseases and encourage regular check-ups in association with professional preventive programs. In particular, this investigation focused on the factors that discourage persons from attending regular check-ups and strategies to overcome them. We also focused on the difference in characteristics between persons who had regular check-ups and those who did not. In this study, we selected dental clinics that routinely perform regular check-ups and provide professional preventive programs in Japan.

Materials and Methods

Study population

Thirty-nine private dental clinics in 15 prefectures throughout Japan participated in this study. These private dentists were members of study groups of the Japan Health Care Dental Association. The questionnaires were distributed over a set period of time to all visiting patients in the waiting room of the dental clinics. Patients returned the questionnaires to the study group center using a postage-paid return envelope. A total of 9,024 questionnaires were distributed, of which 5,132 were returned (response rate 56.8 %). The sample population comprised 1,901 men (37.0%), 3,044 women (59.3%) and 187 unknown (3.6%). It should be noted that the samples surveyed in this study comprised of patients visiting dental clinics where regular check-up systems are in place.

Table 1 Questionnaires used in this study

Gender		Age (years)		Occupation		Household income per month (yen)	Regular check-ups experience	Desired cost for regular check-ups (yen)	
1	Male	1	- 9	1	Office workers	1	less than 200000	Yes	1	1000
2	Female	2	10 - 14	2	Civil servant	2	200000-300000	No	2	2000
		3	15 - 19	3	Self-employed	3	300000-400000		3	3000
		4	20 - 29	4	Housewife	4	400000-500000		4	4000
		5	30 - 39	5	Student	5	more than 500000		5	5000
		6	40 - 49	6	Part-time job				6	7000
		7	50 - 59	7	No occupation				7	10000
		8	60 - 69	8	Others				8	20000
		9	70 -							

Reasons for visiting clinic		Reason for selecting dental office		Information for selecting dental office	
1	Tooth or gum disease	1	Location	1	Specialist
2	Denture problem	2	Personal dental office	2	Technological assessment
3	Health care	3	Technical competence of dentist	3	Cost of treatment
4	Trauma	4	No waiting time	4	Good reputation
5	Others	5	Explanation of treatment	5	Staff
		6	Office open late or on holidays	6	Background of the dentist
		7	Recommendation from associate	7	Others
		8	Recommendation from doctor*		

"Regular check ups" refers to attendance at the dental office for the purpose of the maintaining a healthy oral condition.
* Doctor refers to a medical doctor or another dentist.

Table 2 Demographic characteristics of participants who make regular or infrequent visits for check-ups

(A)

Gender	Regular visitors		Infrequent visitors		No answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
Male	779	41.0	1096	57.7	26	1.4	1901	100 (37.0)
Female	1520	49.9	1475	48.5	49	1.6	3044	100 (59.3)
Unknown	83	44.4	89	47.6	15	8.0	187	100 (3.6)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(B)

Age (years)	Regular visitors		Infrequent visitors		No answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
- 9	23	69.7	10	30.3	0	0	33	100 (0.6)
10 - 14	142	70.6	56	27.9	3	1.5	201	100 (3.9)
15 - 19	32	31.7	68	67.3	1	1.0	101	100 (2.0)
20 - 29	128	35.2	232	63.7	4	1.1	364	100 (7.1)
30 - 39	331	47.5	364	52.2	2	0.3	697	100 (13.6)
40 - 49	406	51.9	376	48.0	1	0.1	783	100 (15.3)
50 - 59	435	44.7	530	54.5	8	0.8	973	100 (19.0)
60 - 69	385	42.4	492	54.1	32	3.5	909	100 (17.7)
70 -	202	35.6	339	59.7	27	4.8	568	100 (11.1)
No answer	298	59.2	193	38.4	12	2.4	503	100 (9.8)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(C)

Occupation	Regular visitors		Infrequent visitors		No Answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
Office worker	412	38.6	643	60.3	11	1.0	1066	100 (20.8)
Civil servant	139	55.4	111	44.2	1	0.4	251	100 (4.9)
Self-employed	205	45.3	238	52.5	10	2.2	453	100 (8.8)
Housewife	627	50.2	598	47.9	23	1.8	1248	100 (24.3)
Student	349	60.0	227	39.0	6	1.0	582	100 (11.3)
Part-time job	181	45.4	215	53.9	3	0.8	399	100 (7.8)
Unemployed	241	37.4	388	60.2	15	2.3	644	100 (12.5)
Others	116	46.6	130	52.2	3	1.2	249	100 (4.9)
No answer	112	46.7	110	45.8	18	7.5	240	100 (4.7)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(A) Gender. (B) Age group. (C) Occupation.
All of these factors were correlated with the likelihood of attending regular check-ups, based on chi-square tests (P values were all less than 0.001). All the chi-square tests excluded "No answer," "Undetermined," and "Unknown" categories. "Regular visitors" refers to participants who answered yes to "Regular check-up experience". "Infrequent visitors" refer to those answering no to "Regular check-up experience".