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THE EFFECTS OF ARECOLINE ON THE RELEASE OF CYTOKINES USING CULTURED PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH ORAL MUCOUS DISEASES

Han-Jen Hsu, Kee-Lung Chang*, Yi-Hsin Yang** and Tien-Yu Shieh**

In Taiwan there is a significant correlation between oral precancer diseases and oral cancer associated with the betel quid chewing habit. The carcinogenic components of betel quid are arecoline, arecaidine and safrole. However, it is unknown whether these substances influence the immune functions. This study investigated the effects of betel quid on the immune system using cultured peripheral blood mononuclear cells from patients with oral mucous diseases. In our experiment, mononuclear cells from 10 normal persons, 12 patients with precancer lesions, and 16 patients with squamous cell carcinoma were separated from blood samples and cultured. After stimulation by arecoline, the amounts of IL-2, TNF- α, TGF- β and IFN- γ secreted by mononuclear cells were measured using the ELISA method. The results showed that IL-2, TNF- α, and TGF- β were significantly lower in mononuclear cells of normal persons as stimulated by arecoline . The TGF- β amount in cells from oral submucous fibrosis patients with betel quid chewing habit (OSF-B) was lower than normal persons or patients who had long term betel quid chewing habit but were without oral mucosal diseases (N-B), and was also lower than the squamous cell carcinoma with betel quid chewing group (SCC-B). TNF- α was significantly lower in the squamous cell carcinoma with long term betel quid chewing group (SCC-B) than in normal persons. TNF- α was significantly higher in the squamous cell carcinoma without betel quid chewing group (SCC-N) than in normal persons and SCC-B groups. In addition, IFN- γ was significantly lower in patients who had long term betel quid chewing but were without oral mucous lesions than the normal person and the OSF group. The results proved that betel quid influences cytokines production by mononuclear cells.

Key words: areca/betel quid, oral submucous fibrosis, SCC, IL-2, TNF- α, TGF- β and IFN- γ

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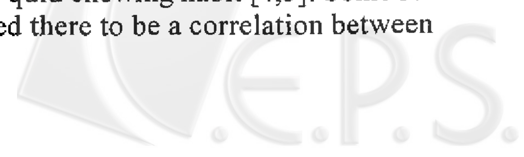
Previous studies have shown that oral mucousal disease is closely related to the betel quid chewing habit in Taiwan [1-3]. According to an earlier study from Kaohsiung Medical University, only 7.6% of all oral cancer patients who do not smoke, drink, or chew betel quid in Taiwan [4,5]. The average age of oral carcinoma patients do not smoke, drink or chew betel quid is close to 60 years old. However, the average age of oral carcinoma patients with the betel quid chewing habit is 10 years younger. The five-year-survival rate was poorer in oral carcinoma patients with the betel quid chewing habit than those without the betel quid chewing habit [4,5]. Some researchers believed there to be a correlation between

Graduate Institute of Dental Sciences,* Graduate Institute of Biological Chemistry, **Graduate Institute of Oral Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan
Received: December 26,2000 Accepted: April 9, 2001
Address for reprints: Tien-Yu Shieh, Graduate Institute of Oral Health Sciences, No.100, Shih-Chuan 1st Rd., Kaohsiung Medical University, Kaohsiung 807, Taiwan



the immune system and oral mucous disease. The mucous fibrosis characteristics of the OSF was similar to the immune disease – Scleroderma. Inflammation tissue and immune disease were related to cytokine. Cytokine can increase the immune ability for the host to avoid the invasion of the micro-organic and to prevent the growth of the tumor cell. Cytokine can induce the function of the inflammation reaction and the ability of the tissue repair, but it will cause immune disease if the cytokine becomes unbalanced. Increasing of the cytokine activity and an unbalanced cytokine network may cause immune disease.

IL-2 that is produced by T lymphocytes stimulates the proliferation of B cell, T cell and NK cell and produces the lymphokine. This will increase the reaction of the NK cytokine. Immune inhibitor of the SCC p't can be removed by IL-2 [6]. Therefore, we can understand the activity of T cell by detecting IL-2. We can also know whether the betel quid components influence the activity of T cell.

Tumor necrosis factor (TNF) was improved to the effect that it could kill the tumor cell. TNF can stimulate T cell to produce cytokine. This study also aims to ascertain whether the betel quid components stimulate the produce of TNF.

Interferon (IFN) α, β and γ can influence the cell change, such as cell proliferation, differentiation and they can control the immune system. We can measure the concentration of IFN-γ to detect the strength of the immune system.

Measurements of cytokine secretion are used for the determination of cellular immunity status. There were significantly lower values of INF-γ and IL-2, and the levels of IL-1 and TNF-α were reduced in patients with melanoma [7]. Each serum cytokine seems to reflect a characteristic pathology of individual oral disease. TGF-β has an effect on collagen synthesis [8,9]. Human buccal mucosa fibroblasts in vitro were stimulated by betel nut alkaloids in different concentrations. However no research was done about the relationship between cytokine level and betel quid.

Hence, this study was undertaken to assess the immune status using changes in the amount of cytokines under stimulation by one of the components of betel quid - arecoline.

MATERIALS AND METHODS

Patients

This study was conducted using 12 oral submucous fibrosis (OSF) patients and 16 oral squamous cell carcinoma (SCC) patients. Ten patients with squamous cell carcinoma had a long-term betel quid chewing habit (SCC-B). The other six patients had no such history (SCC-N). Ten patients from the oral submucous fibrosis group had a long-term betel quid chewing habit (OSF-B) while the other two patients did not (OSF-N). In order to elaborate the possible roles of betel quid chewing habits and host factors on cytokine production of mononuclear cells, ten subjects had long-term betel quid chewing habits but were without any oral mucous lesions (N-B, betel quid 30 ± grams/day over 20 years)and 10 healthy normal adult controls, with no oral mucous diseases and no betel quid chewing (N) were also included. All of the subjects were screened clinically to exclude the possibility of any other medical factors.

Blood samples and mononuclear cell cultures

Twenty ml of peripleral blood was collected from all subjects using preservative-free heparin. The heparinized blood was diluted 1:1 with Hank's balanced salt solution (HBSS) and layered on to Ficoll-Hypaque. After centrifugation at 400Xg for 20 minutes, the mononuclear cell interface was carefully collected and washed twice in 20ml of HBSS and centrifugated for 10 minutes at 400Xg. The mononuclear cells were suspended at a concentration of 1×10^6 cells /ml in RPMI 1640 medium containing 10% heat-inactivated (56℃, 30min) fetal bovine serum (FBS, Gibco company), 1% streptomycin, and 1% penicillin. Incubation of the cell cultures was performed at 37℃ in a humidified atmosphere of 5% carbon dioxide. After 3 days (72 hours) of culture without a change in medium, 1ml of supernatant was removed from each tube after being centrifuged at 400Xg for 10 min. The supernatant was then frozen at -20℃ until assay for cytokine levels [10].

Choose arecoline stimulating concentration

The mononuclear cells of subjects with normal oral mucosa who were not betel quid chewers were incubated for 72 hours with culture medium exposed to arecoline (C8H13NO2HBr: Methy 1-methyl-1,2,5, 6-terahydronicotinate, FW: 236.1. Sigma.A-6134) at concentrations of 1 μg/ml, 10 μg/ml, 30 μg/ml, 50 μg/ml, 70 μg/ml, 100 μg/ml, 150 μg/ml, and 200 μg/ml. Stimulation was more pronounced with 10 μg/ml, indicating a concentration-dependent effect. A concentration of 10 μg/ml arecoline was therefore added [11-14].

Determination of cytokine levels

Highly sensitive, specific, and reproducible enzyme-linked immunosorbent assays (ELISA) were applied for qualitative and quantitative determinations of IL-2, TNF- α , TGF- β and IFN- γ . These tests were based on the sandwich principle and were performed in one step. ELISA kits were purchased from Quantikine immunoteck S.A. Assay procedures were carried out according to the manufacturer's instructions [15,16].

ELISA kits of IL-2 contained E. coli-expressed recombinant human IL-2 and antibodies raised against recombinant human IL-2. The sensitivity range is 7-2000 pg/ml. Each TNF- α kit contained E. coli-expressed recombinant human TNF- α and antibodies raised against this protein. The ELISA sensitivity range is 4.4-1000 pg/ml. The kit of IFN- γ contained E. coli-expressed recombinant human IFN-g and antibodies raised against recombinant human IFN- γ . The ELISA sensitive range is 3.0-1000 pg/ml. Kits contained recombinant human TGF- $\beta 1$ expressed by CHO cells and have been shown to quantitate the recombinant factor. The ELISA sensitivity range is 7-2000 pg/ml.

Statistical analysis

The nonparametric statistical methods were used in ths analysis for considering the small sample size. Wilcoxon /Kruskal-wallis test (Rank Sums) was used to assess the significance of differences between the cytokine averages in different disease patients with or without a betel quid chewing habit. Signed-Rank test was used to assess the significance of differences between the cytokine means in different disease patients with or without added arecoline.

RESULTS

The cytokines releases by cultured peripheral blood mononuclear cells from normal persons and from patients with oral mucosal diseases are shown in Table 1. Considering each lesion/habit group separately, the arecoline stimulation was significantly lower than the control groups for all cytokines measured in the normal oral mucosa group from persons with no betel-quid chewing experience (N), and the group of OSF from patients with no betel-quid chewing experience (OSF-N). It was found that the arecoline stimulation was higher in IL-2 and IFN- γ , and the TNF- α and TGF- β measures were opposite in the group of OSF from betel-quid chewers, the SCC group without chewing experience, and the SCC group from betel-quid chewers except the OSF with chewing habit group (OSF-B)($p<0.05$).

For OSF patients, persons with betel-quid chewing habit had higher TNF- α , but lower IL-2, TGF- β and IFN- γ measures. For SCC patients, betel-quid chewers had lower TNF- α , TGF- β and IFN- γ (arecoline stimulated), and higher IL-2 and IFN- γ (control). When considering the N group as a reference, the OSF-N and OSF-B were both significantly lower than in cytokines, except that TNF- α was opposite. For the TNF- α measures, the N group fell between SCC-N and SCC-B, and the differences were all significant. For betel-quid chewers, the IL-2 (control), TGF- β and IFN- γ from normal patients were greater than those of the SCC , and OSF patients, while the level of TNF- α was OSF> no-lesion>SCC (Table 2).

DISCUSSION

This study used IL-2, TNF- α , TGF- β and IFN- γ producing ability of the peripheral blood mononuclear cell to determine the level of immune ability (strong or weak)[17-21]. In persons with normal oral mucosa and betel quid chewing experience, the amounts of IL-2, TNF- α, and TGF- β decreased after stimulation with arecoline, while the amount of IFN- γ showed no change. This revealed that arecoline can inhibit the IL-2, TNF- α , and TGF- β releasing ability of the peripheral blood monocytes, and it could influence the immune ability. Some betel quid chewers often said that they had no oral disease even after long-term chewing and they did not want to stop this habit. This study specifically examined the peripheral blood of N-B patients (no oral mucous disease, long-term betel quid chewing habit), and found a significantly lower IFN- γ level than in normal persons. Although the TGF- β of N-B decreased after adding arecoline, it was still higher than the TGF- β amount in a normal persons after adding arecoline (Table 1). The IL-2, TGF- β and IFN- γ of N-B were also higher than the amounts in OSF-B/SCC-B patients (Table 2). According to previous studies of TGF- β immune inhibition ability, some authors found that the lymphocyte stimulated by TGF- β will influence the messenger transfer between IL-2 and its receptor. This will then decrease the amount of IFN- γ [22,23]. IFN- γ is one of the most important cytokines for inducing inflam-

Table 1. Comparison of cytokines released by cultured peripheral blood mononuclear cells from normal persons and from patients with oral mucosal diseases

Cytokines (pg/ml) group	Normal oral mucosa from persons with no betel-quid chewing experience (N) (n=10)	Betel-quid chewers without any oral mucosal lesions (N-B) (n=10)	N vs. N-B	OSF from persons with no betel-quid chewing experience (OSF-N) (n=2)	OSF from betel-quid chewers (OSF-B) (n=10)	OSF-N vs. OSF-B	SCC from persons with no betel-quid chewing experience (SCC-N) (n=6)	SCC from betel-quid chewers (SCC-B) (n=10)	SCC-N vs. SCC-B
IL-2									
Control	77.18 ± 4.93	83.83 ± 9.27		56.67 ± 2.14 &	23.76 ± 4.27 &	*	33.79 ± 9.20 &	39.8 ± 4.86 &	
AL	70.35 ± 3.09	83.53 ± 10.58		55.07 ± 0.00 &	38.68 ± 6.17 &		48.98 ± 5.7	181.38 ± 94.86 &	
AL vs. Control	*								
TNF-α									
Control	48.12 ± 4.04	45.98 ± 4.41		50.68 ± 5.06 &	128.03 ± 32.76		222.15 ± 70.69 &	24.55 ± 4.78 &	*
AL	40.07 ± 4.1	50.59 ± 2.87		46.21 ± 4.39 &	123.16 ± 32.13	*	213.47 ± 66.47 &	19.19 ± 3.19 &	*
AL vs. Control	*	*		*	*				
TGF-β									
Control	1181.52 ± 196.06	1704.48 ± 154.25		635.64 ± 1.62 &	550.3 ± 33.66 &		953.58 ± 13.35	911.52 ± 81.94	
AL	1087.66 ± 176.32	1656.9 ± 138.41 &	*	606.59 ± 1.61 &	474.7 ± 26.04 &	*	896.32 ± 50.02	871.56 ± 67.77	
AL vs. Control	*	*		*	*			*	
IFN-γ									
Control	86.02 ± 9.75	49.06 ± 8.20 &	*	27.47 ± 2.45 &	15.89 ± 1.20 &		17.92 ± 3.65 &	21.14 ± 1.33 &	
AL	63.24 ± 10.09	41.31 ± 4.75		26.81 ± 2.45 &	18.47 ± 1.82 &		25.03 ± 3.90 &	24.63 ± 1.98 &	
AL vs. Control				*			*		

AL : arecoline

* : p-value<0.05 by wilcoxon rank sum tests.

Table 2. Comparison of cytokines released by cultured peripheral blood mononuclear cells among betel-quid chewers

Cytokines (pg/*ml*) group	Betel-quid chewers without any oral mucosal lesions (N-B) (n=10)	OSF from betel-quid chewers (OSF-B) (n=10)	SCC from betel-quid chewers (SCC-B) (n=10)	OSF-B vs. N-B	SCC-B vs. OSF-B	SCC-B vs. N-B	Cytokines titer sequence
IL-2							
Control	83.83 ± 9.27	23.76 ± 4.27	39.8 ± 4.86	*	*	*	N-B > SCC-B > OSF-B
AL	83.53 ± 10.58	38.68 ± 6.17	181.38 ± 94.86	*			
TNF-α							
Control	45.98 ± 4.41	128.03 ± 32.76	24.55 ± 4.78	*	*	*	OSF-B> N-B > SCC-B
AL	50.59 ± 2.87	123.16 ± 32.13	19.19 ± 3.19		*	*	
TGF-β							
Control	1704.48 ± 154.25	550.3 ± 33.66	911.52 ± 81.94	*	*	*	N-B > SCC-B > OSF-B
AL	1656.9 ± 138.41	474.7 ± 26.04	871.56 ± 67.77	*	*	*	N-B > SCC-B > OSF-B
IFN-γ							
Control	49.06 ± 8.20	15.89 ± 1.20	21.14 ± 1.33	*	*	*	N-B > SCC-B > OSF-B
AL	41.31 ± 4.75	18.47 ± 1.82	24.63 ± 1.98	*	*	*	N-B > SCC-B > OSF-B

AL : arecoline

* : p-value<0.05 by wilcoxon rank sum tests.

mation reactions. This study found that N-B patients had higher TGF-β and lower IFN-γ than OSF-B/SCC-B patients (Table 1,2). If the IFN-γ producing ability was inhibited, inducing an inflammation reaction will become more difficult. This may be one of the reasons that even after long-term betel quid chewing, they still had no oral mucous disease.

Generally speaking, OSF is always induced by betel quid chewing habit. Other evident etiology is very limited. We had only 2 cases in the KMU hospital with no history of chewing betel quid. In those cases, OSF was caused by eating hard / abrasive food. Perhaps the mechanical abrasion is the etiology. IL-2 amounts in the OSF-B group were lower than OSF-N patients (TNF-α, TGF-β and IFN-γ had no significant differences)(Table 1). This result reveals that even the same oral mucous diseases had different cytokines expression with different etiologies. Our previous studies found that there were larger amounts of TGF-β in the lesion tissue of OSF-B patients than in normal oral mucosa tissue [24]. TGF-β also has these functions in that it can induce fibroblast production and inhibit collagenase ability [8,9,25]. TGF-β is an important etiology in OSF in local tissue. In this study, the TGF-β amount in OSF-B patients in peripheral blood was lower than in normal persons, N-B persons and SCC-B patients (Table 1). With or without betel quid chewing, OSF-N / OSF-B patients had lower IL-2, TGF-β and IFN-γ than normal persons, and the TNF-α amount in OSF patients was higher than in normal persons (Table 1). Even though TNF-α amount in OSF-B patients showed no significant difference to N persons, it was still the highest in OSF-B, SCC-B and

N-B persons (Table 2).

Nearly all patients with oral cancer in Taiwan have betel quid chewing experience [4]. The SCC patients who did not chew betel quid were very few [4]. We had no more than 10 cases per year in our hospital. This study had only 6 cases of oral squamous cell carcinoma who did not chew betel quid. The TNF-α amount in SCC-N patients was higher than in normal persons (Table 2). Aside from TNF-α, the other cytokines (IL-2, TGF-β and IFN-γ) showed no significant differences between betel quid chewers or non-betel quid chewers. In SCC-N patients, their IL-2, TGF-β and IFN-γ were lower than normal persons (Table 1). TNF-α amounts will elevate to deflect cancer cell formation. SCC-N patients had higher TNF-α levels, which reveals that the TNF-α production function by the T lymphocytes still existed in their body. The TNF-α level of SCC-B patients was lower than SCC-N patients and even lower than normal persons. After stimulation with arecoline, the TGF-β levels of SCC-B patients decreased and the IFN-γ levels of SCC-N patients also decreased. There is a different expression of TGF-β and IFN-γ between SCC-N and SCC-B patients after stimulation with arecoline (Table 1). We can confirm that betel quid indeed influences the cytokine production of mononuclear cells.

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檳榔素對於有嚼食檳榔習慣之黏膜下纖維化及鱗狀上皮細胞癌患者在周邊血液單核球培養中細胞激素生成影響之研究

許瀚仁 張基隆* 楊奕馨** 謝天渝**

在台灣許多口腔黏膜疾病和嚼食檳榔習慣有很大的關連。檳榔塊的組成中被認為主要的致癌部分是檳榔素(arecoline, AL)，檳榔鹼(arecaidine, AD)，以及荖花中的黃樟素(safrole,S)，此類物質是否影響免疫反應，至今無人瞭解。因此本研究欲探討口腔黏膜疾病和口腔癌病患其免疫細胞分泌之各類細胞激素是否會受檳榔塊成份的影響。實驗中分離正常人及病人血液中之單核球加以培養，以檳榔素成份刺激後，測量IL-2、TNF-α、TGF-β、IFN-γ等和細胞免疫相關細胞激素的量。結果顯示，長期嚼食檳榔而口腔黏膜仍正常者的單核球產生TGF-β的量大於未嚼食檳榔且口腔黏膜正常者；當加入檳榔素(arecoline,AL)後，未嚼食檳榔且口腔黏膜正常者的單核球產生IL-2、TNF-α、TGF-β的量皆下降。由具正常口腔黏膜但有嚼食檳榔習慣者與具正常口腔黏膜無嚼食檳榔習慣者比較各種細胞激素的生成，發現IL-2及TNF-α的生成上沒有顯著差異。IFN-γ的生成量，無嚼食檳榔者較嚼食檳榔者的量高（$p < 0.02$）。OSF的患者除TNF-α明顯高於正常人及SCC的患者外，IL-2、TGF-β、IFN-γ皆較低。尤其是TGF-β分泌的量比正常人或SCC的患者低甚多。另外，從本研究也發現長期嚼食檳榔而仍具正常口腔黏膜者有大量的TGF-γ。TGF-β抑制IFN-γ之生成及發炎反應，可能是雖長期嚼食檳榔而仍有正常口腔黏膜的原因。從本研究得知，雖然皆爲口腔鱗狀上皮細胞癌，但因長期嚼食檳榔而得口腔癌者與無此習慣而得口腔癌者之間其單核球細胞產生IL-2、TNF-α、TGF-β、IFN-γ 等四種細胞素的量不盡相同。兩者在IL-2、TGF-β及IFN-γ的生成雖沒有差異，但具嚼食檳榔習慣得口腔癌（SCC）者的TNF-α生成量比正常者低。而無此習慣而得口腔癌者的TNF-α則比正常者或嚼食檳榔得口腔癌者都高出甚多。嚼檳榔得口腔癌者與無嚼檳榔得口腔癌者之單核球細胞在加入檳榔素後，兩者之TNF-α表現不同。由此可知嚼食檳榔確實會影響檳榔素對免疫細胞之細胞素的分泌，而SCC與OSF的致病機轉可能因這些細胞激素表現上有明顯差異而有所不同。

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高雄醫學大學 牙醫學研究所
* 高雄醫學大學 生物化學研究所
** 高雄醫學大學 口腔衛生科學研究所
收文日期:89年12月26日 接受刊載:90年4月9日
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