Biotherapy
バイオセラピー

Antitumor Effect of Hot-Water Extract of TAHEEBO Tea
− Comparison with Other Biological Preparations −

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Summary
The antitumor effect of TAHEEBO tea extract was examined in terms of its direct action on tumor cells and inhibitory action on tumor angiogenesis. Early induction of apoptosis was assayed by the binding of Annexin-V and phosphatidyl serine (PS) in the cellular membrane. TAHEEBO tea extract induced apoptosis of human Daudi and Baji B lymphoblastoid cells. Treatment with TAHEEBO tea extract also inhibited the in vitro growth of Daudi and Raji cells. TAHEEBO tea extract also inhibited the growth of human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC). These results indicate that TAHEEBO tea extract has diversified antitumor functions, ie enhancement of sequential immune mechanisms, a direct cytotoxic effect and an inhibitory action on angiogenesis in tumors. The antitumor effect of naphthoquinone, purified from TAHEEBO tea extract, was examined in a double grafted tumor system〒 of BALB/c mice. Based on this evidence, we propose what can be called 〆integrative medicine〒 which is neither Western nor Chinese medicine.

Key words: Biological response modifier (BRM), TAHEEBO tea, integrative medicine, Apoptosis, Angiogenesis
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Introduction

We have clarified that some biological preparations (i.e., biological response modifiers, BRMs hereinafter), when administered into primary tumors, cure not only the primary tumors, but also distant metastatic tumors, using a "double grafted tumor system" in mice, which we have devised[1-6]. In other words, a tumor limited to the site of origin is unproblematic because it can be surgically removed, whereas in the presence of distant metastatic tumors, particularly in the presence of micrometastatic tumors that are not visible macroscopically, the surgical removal of the primary lesion leads to the growth of metastatic tumors but does not lead to cancer treatment. Thus, the treatment of metastatic lesions is a major issue in cancer treatment.

In the previously reported study, we clarified the antimetastatic effect of the hot-water extract of TAHEEBO (TAHEEBO tea) in a "double grafted tumor system," in a spontaneous lung metastasis model and in a spontaneous liver metastasis model[7]. TAHEEBO tea contains naphthoquinone as the major ingredient and is widely consumed in Brazil, South America. The TAHEEBO tea used is a hot-water extract of the bark of a Bignoniaceae plant (botanical name: Tabebuia avellanedae), and its major ingredient is naphthoquinone[8]. Ueda et al. has reported that a naphthoquinone compound purified from TAHEEBO tea suppresses the TPA-induced activation of the early antigen expression of EB virus and possesses in vitro antitumor-promotion activity[9]. In the present study, we investigated the antitumor effect of the purified naphthoquinone, as well as the direct action of the TAHEEBO tea extract on tumor cells and its inhibitory action on angiogenesis, in comparison with other BRMs.

Ⅰ. Materials and methods

1. Mice and tumor cells

BALB/c male mice obtained from Japan SLC Inc. were used at 7 weeks of age. Meth-A fibrosarcoma cells from the same strain of BALB/c mice were inoculated subcutaneously as a solid tumor.

2. Study substances

The TAHEEBO tea extracts were prepared using a bark tea TAHEEBO, which was kindly provided by TAHEEBO JAPAN Co., Ltd. The bark tea TAHEEBO uses the pure inner bark of Tabebuia avellanedae of Bignoniaceae, which was collected by the Nogueira Chagas Company, Brazil, as the raw material. The hot-water extracts of the TAHEEBO tea were prepared as follows. To 900 mL of water, 15 g of TAHEEBO tea was added, and it was boiled for either 5 minutes or 30 minutes. These two types of hot-water extracts were used in the experiment. The 5-min boiled hot-water extract and the 30-min boiled hot-water extract contain 25 and 75 mg/mL of naphthoquinone, respectively. In addition, as a purified compound from TAHEEBO tea, 25 and 1 mg/mL of naphthoquinone were used in the experiment.

3. Double grafted tumor system

BALB/c mice received simultaneous intradermal inoculations of Meth-A cells on the right flank (10^6 cells) and left flank (2×10^5 cells). From Day 3 when the large tumor (assumed to be the primary lesion) on the right flank became palpable, the drugs (0.1 mL) were administered intratumorally for 3 consecutive days to cure the tumor. The untreated left-flank distant tumor (assumed to be a metastatic lesion) was observed for shrinkage.

4. Detection of apoptosis

Asymmetry of the phospholipid bilayer in cell membranes is lost in the early phase of apoptosis. Phosphatidylserine (PS) is normally located in the inner leaflet of cell membranes. However, upon the induction of apoptosis, PS is translocated to the outer surface of the membrane without disruption of the membrane structure. Since FITC-labeled Annexin V binds to PS with high affinity, apoptosis was detected by the binding, using FACScan (MEBOCYTO Apoptosis Kit, MBL)[10].

5. Measurement of cell growth capacity

Assays using a tetrazolium salt have become available for the measurements of cell growth capacity and viability. A tetrazolium salt exists in the mitochondrial respiratory chain, and is degraded into formazan dye by succinate-tetrazolium reductase, which is active only in viable cells. Because the enzyme activity increases as the number of viable cells increases, the increase in formazan production was measured as the absorbance of the dye solution, using an ELISA reader (Premix WST-1, by Takara)[11].

6. Inhibitory action on vascular endothelial cell growth

The growth inhibition of the TAHEEBO tea extracts was investigated using normal human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC).

Ⅱ. Experimental results

1. Apoptosis induction of TAHEEBO tea extracts

Early apoptosis induction of the 5-min and 30-min extracts of TAHEEBO tea was measured after 24-hr incubation, by flow cytometry (FACScan). As shown in the positive control in Fig. 1, the lower right-hand part of the FACScan graph shows the distribution of the percentage of PS positive cells bound to Annexin V, which are considered to have induced early apoptosis. In both of the Daudi and Raji human tumor cells, as compared with the control, the induction of apoptosis was observed in the group treated with the 30-min extract of TAHEEBO tea. On the other hand, in normal peripheral blood lymphocytes, the TAHEEBO tea extract did not induce apoptosis.

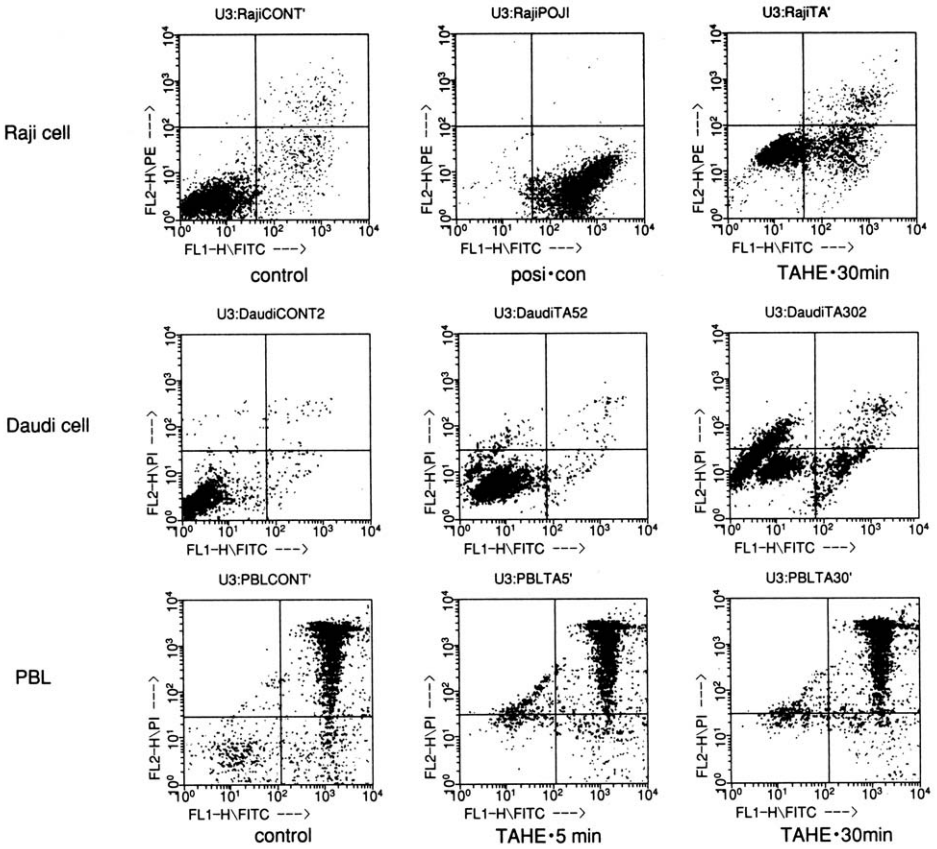

Fig. 1 Apoptosis induction by TAHEEBO

2. Inhibitory action of TAHEEBO tea extracts on cell growth

The inhibitory action of TAHEEBO tea extracts on cell growth was measured by directly counting the cell numbers of both the Daudi and Raji human tumor cells. The result showed the inhibitory action of the extracts on cell growth, as shown in Fig. 2. Then, the number of viable cells was measured using an enzyme. As shown in Fig. 3, the growth of tumor cells was inhibited, but no effect on normal peripheral lymphocytes was observed.

3. Inhibitory action of TAHEEBO tea extracts on vascular endothelial cell growth

The effect of the 5-min and 30-min hot-water extracts of TAHEEBO tea on vascular endothelial cells was investigated. As shown in Fig. 4, a distinct growth inhibition of both HUVEC and HMVEC was observed.

4. Antitumor effect of the purified naphthoquinone from TAHEEBO tea in the "double grafted tumor system"

Naphthoquinone that is the active ingredient of the TAHEEBO tea extracts was purified and investigated for its antitumor effect in the "double grafted tumor system." As shown in Fig. 5, the purified naphthoquinone showed little antitumor effect.

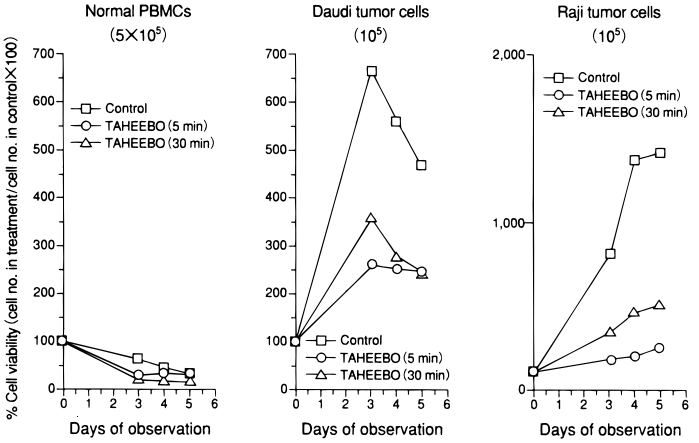

図 2 Direct *in vitro* cytotoxicity of TAHEEBO

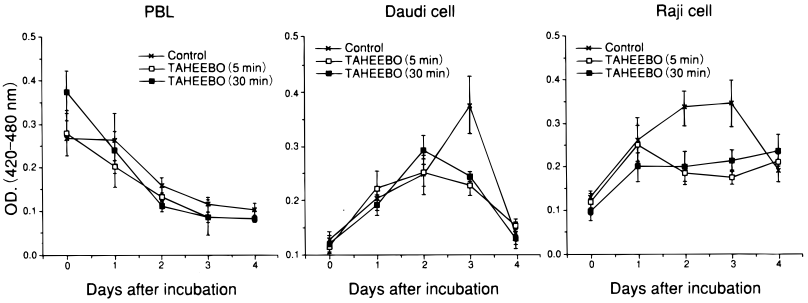

Fig. 3 Inhibitory effect of TAHEEBO tea extracts on tumor cell growth

Ⅲ. Discussion

In the previously reported study[7], we found that the TAHEEBO tea extract inhibits the growth of the left and right tumors in the "double grafted tumor system" and activates macrophages through the induction of serum IAP. In addition, we clarified that the TAHEEBO tea extract inhibits the invasion of tumor cells by directly acting on tumor cells.

In the present study, we clarified that the TAHEEBO tea extract induces apoptosis in tumor cells but not in normal lymphocytes (Fig. 1), and inhibits the growth of vascular endothelial cells (Fig. 4). However, compared with other BRMs (PSK[4)6)] and cepharanthine[4)12)]), the TAHEEBO tea extract is found to have slightly different actions, as shown in Table 1.

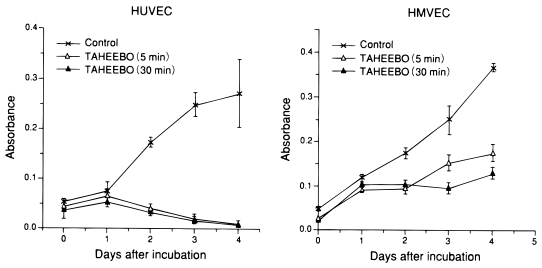

Fig. 4 Inhibitory effect of TAHEEBO tea extracts on vascular endothelial cell growth

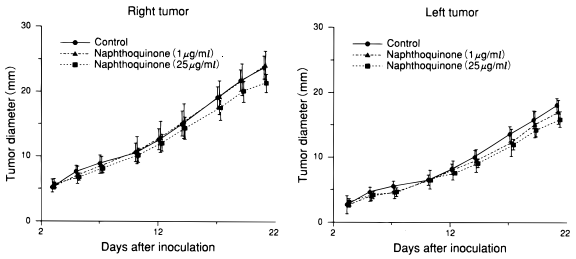

Fig. 5 Antitumor effect of naphthoquinone

表 1 Comparison of biological activities among biological preparations

Biological preparations	Anti-metastatic effect in "double grafted tumor system"	Inhibition of tumor cell invasion	Induction of apoptosis		Growth inhibition of vascular endothelial cells	
			Tumor cell	PBL	HUVEC	HMVEC
PSK	++	+	±	−	−	−
Cepharanthin	+	+	++	±	+	±
TAHEEBO tea	+	+	+	−	+	+

Then, the TAHEEBO tea extract was compared with the BRMs studied so far, in terms of the antitumor effect determined in the "double grafted tumor system" and the macrophage activation capacity measured based on serum immunosuppressive acidic protein (IAP) levels. As shown in Table 2, PSK (a basidiomycete extract), cepharanthine (a herbal medicine), and TAHEEBO tea (a bark tea extract) have both an antitumor effect and macrophage activation capacity. On the other hand, their purified compounds, which are b-glucan, alkaloid, and naphthoquinone, respectively, have been found to have a weaker antitumor effect and macrophage activation capacity. This result indicates that extracts are more likely to be recognized by macrophages than purified products, and that the antitumor effect is exerted through the antitumor immunological cascade reactions.

表 2 Anti-metastatic effect and macrophage activation of biological preparations

Biological preparation	Double grafted tumor system				IAP induction in serum ($\mu g/ml \pm SD$)
	Primary tumor		Metastatic tumor		
	Tumor free/ tested	Tumor weight (g±SD)	Tumor free/ tested	Tumor weight (g±SD)	
Extract of *Coliolus versicolor*, Protein bound β-glucan (PSK)	4/8	0.7±0.52	2/8	1.4±0.74	930±77
Purified β-glucan from *Lentinus edodes* (LNT)	0/9	5.8±1.0	0/9	3.7±0.6	189±42
Extract of *Stephania cepharantha* HAYATA (CR)	3/8	1.2±0.6	2/8	2.5±0.88	840±62
Purified alkaloid, cepharanthine	5/10	1.3±2.7	0/10	3.2±0.97	320±34
Extract of TAHEEBO tea	3/7	1.1±1.4	1/7	1.9±0.9	452±58
Purified naphthoquinone	0/6	3.7±0.8	0/6	1.6±0.2	174±22
Control, saline	0/8	5.3±0.72	0/8	3.1±0.5	133±18

SD : standard deviation

The drugs used in conventional Western medicine are purified compounds, which have specified chemical structures, whereas the drugs used in traditional Chinese medicine are Chinese herbal medicines, which are mixtures of several herbal drugs. However, purified compounds act as a poison when used at the wrong doses and are always accompanied by the risk of adverse reactions, resulting in cases of medical malpractice.

On the other hand, a drawback in the use of Chinese herbal medicines is that the effective compound cannot be identified. This poses a risk of adverse reactions when Chinese herbal medicines are used concomitantly with Western medicines. Under such circumstances, we have devised "integrative medicine," which is neither Western nor traditional Chinese medicine13). The drugs used in integrative medicine refer to extracts containing active ingredients whose structures and functions have been identified, such as PSK, cepharanthine and TAHEEBO tea extract. In recent years, these extracts have been recognized as functional food and are characterized by fewer and milder adverse reactions.

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バイオセラピー

樹木茶タヒボ抽出物の抗腫瘍効果 ―他生物製剤との比較―

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要旨　樹木茶タヒボの熱水抽出物の腫瘍細胞への直接作用と血管新生抑制作用について検討した。ヒト腫瘍細胞 Daudi と Raji 細胞に対し，水 900 m*l* にタヒボ茶 15 g を加えて 5 分間ならびに 30 分間沸騰させた熱水抽出物を 0.1 m*l* ずつ処理したところアポトーシスを誘導し，両細胞の増殖を抑制した。一方，正常末梢リンパ球に対してはアポトーシス誘導も増殖阻害もなかった。また，ヒト臍帯静脈血管内皮細胞とヒト皮膚由来微小血管内皮細胞に対する増殖抑制作用がみられた。タヒボ茶抽出物は抗腫瘍免疫増強作用と腫瘍細胞への直接作用ならびに腫瘍血管新生抑制作用という多彩な作用で，腫瘍の増殖を抑制することがわかった。精製物より抽出物のほうが抗腫瘍効果があることから“統合医学”の発想に結び付いた。

はじめに

各種生物製剤（BRM）を原発腫瘍内に投与すると原発腫瘍のみならず，遠隔転移腫瘍まで治癒させるもののあることを，われわれが考案したマウス“二重移植腫瘍系”で明らかにしてきた[1-6]。すなわち腫瘍がまだ原発巣しかない場合，手術して摘出すれば問題はないが，すでに遠隔転移巣がある場合，特に肉眼で確認できない微小転移巣がある場合は，手術によって原発巣を摘出すると転移巣が増殖を開始し，癌治療には結び付かない。すなわち，転移巣の治療が癌治療の大きな課題といえる。

前報で南米ブラジルで広く飲用されているナフトキノンを主成分とする TAHEEBO（タヒボ茶）の熱水抽出物の転移抑制効果について“二重移植腫瘍系”と自然肺転移ならびに肝転移モデルで明らかにした[7]。タヒボ茶はノウゼンカズラ科の学名 *Tabebuia avellanedae* の樹皮の熱水抽出物で，その主成分はナフトキノンである[8]。上田らはタヒボ茶精製物ナフトキノンが TPA 誘発 EB ウイルス初期抗原の活性化の抑制を示し，*in vitro* で抗発癌プロモーター活性を有する成分であることを報告している[9]。今回，精製ナフトキノンの抗腫瘍効果を調べるとともにタヒボ茶抽出物の腫瘍細胞への直接作用，ならびに血管新生抑制作用について検討し，他生物製剤の作用と比較したので報告する。

I.　材料と方法

1.　マウスと腫瘍細胞

日本エスエルシー（株）より購入した 7 週齢 BALB/c 雄マウスを使用した。腫瘍は BALB/c マウスと同系の Meth-A 線維芽肉腫細胞を皮下に接種し固形腫瘍として使用した。

2.　薬　剤

タヒボ茶抽出物はブラジル連邦共和国ノゲイラ・シャーガス社が伐採したノウゼンカズラ科・*Tabebuia avellanedae* の純正内部樹皮を原料とする樹木茶 TAHEEBO をタヒボジャパン（株）より恵与を受け，熱水抽出物を得た。タヒボ茶（TAHEEBO）15 g を 900 m*l* の水に入れ，5 分間沸騰させた熱水抽出物ならびに 30 分間沸騰させた熱水抽出物を実験に供した。5 分間沸騰させた熱水抽出物には 25 μg/m*l* の，30 分間沸騰させた熱水抽出物には 75 μg/m*l* のナフトキノンが含まれていることがわかっている。またタヒボ茶精製物としてナフトキノン 25 μg/m*l* と 1 μg/m*l* を実験に供した。

3.　二重移植腫瘍系

BALB/c マウスの右側腹皮内に 10^6 個，左側腹皮内に 2×10^5 個の Meth-A 細胞を同時に移植し，右側の大きな腫瘍（原発巣と想定）が指で触れるようになる 3 日目より，腫瘍内に薬剤 0.1 m*l* を 3 日間連日投与することにより治療し，治療していない左側の遠隔腫瘍（転移巣と想定）の退縮を観察する系である。

4.　アポトーシス検出

アポトーシスの早期には細胞膜のリン脂質の非対称性が失われることが知られている。phosphatidyl serine（PS）は通常，細胞膜内層に存在しているが，アポトーシスが誘導されると膜の構造は伴ったままで細胞膜外にでてくる。FITC 標識 Annexin V が，高い親和性をもって PS と結合することから，これを FACScan で検出した（MEBOCYTO Apoptosis Kit, MBL）[10]。

5. 細胞増殖能測定

細胞増殖能力や生存能力の測定にテトラゾリウム塩が使われるようになった。テトラゾリウム塩はミトコンドリアの呼吸鎖に存在して，生存細胞にだけ活性のあるコハク酸塩テトラゾリウム還元酵素によりホルマザン色素に分解される。生存細胞数が増加すれば，酵素活性が増加するので，ホルマザン色素の増加をELISAリーダーで色素溶液の吸光度により測定した（Takara，Premix WST-1）[11]。

6. 血管内皮細胞増殖抑制作用

正常ヒト臍帯静脈血管内皮細胞（HUVEC）とヒト皮膚由来微小血管内皮細胞（HMVEC）を使い，タヒボ茶抽出物の増殖抑制作用を調べた。

II. 実験結果

1. タヒボ茶抽出物のアポトーシス誘導能

タヒボ茶5分間抽出物と30分間抽出物を24時間処理した時の初期アポトーシス誘導能をフローサイトメトリー（FACScan）にて測定した。図1

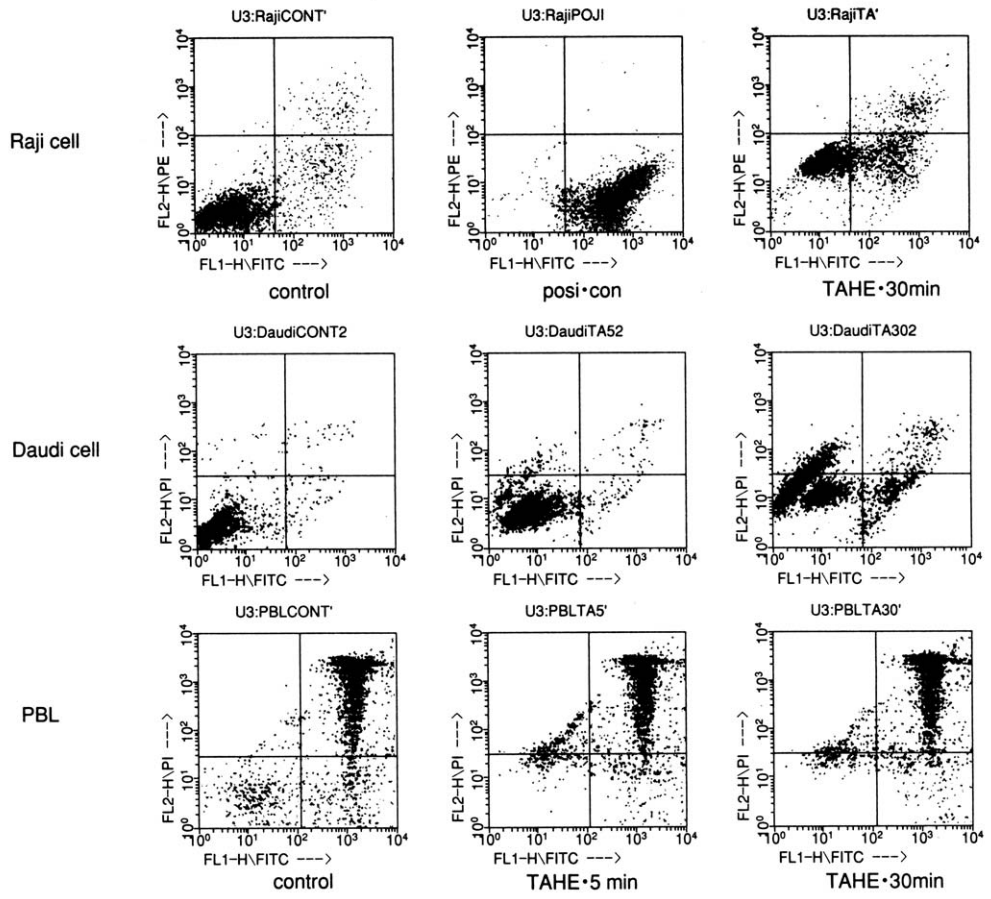

図 1 Apoptosis induction by TAHEEBO

のpositive controlに示すようにFACScan右下のカラムがAnnexin Vと結合したPS陽性の細胞%を表し，初期アポトーシスを引き起こしたものと考えられる。その結果Daudi，Raji両ヒト腫瘍細胞共対照に比し，タヒボ茶30分間抽出物処理群でアポトーシスを引き起こしていた。一方，正常末梢血リンパ球に対してはタヒボ茶抽出物はアポトーシスを誘導しなかった。

2. タヒボ茶抽出物の細胞増殖抑制作用

まず，タヒボ茶抽出物のDaudi，Raji両ヒト腫瘍細胞に対する直接細胞数算定により細胞増殖抑制作用を測定したところ，図2に示すように細胞増殖抑制作用がみられた。次に生存細胞数を酵素により測定したところ，図3に示すように腫瘍細胞の増殖を抑えたが，正常末梢リンパ球への作用はなかった。

3. タヒボ茶抽出物の血管内皮細胞増殖抑制作用

タヒボ茶熱水5分間ならびに30分間抽出物の血管内皮細胞に対する作用を検討した。図4に示すようにヒト臍帯静脈血管内皮細胞（HUVEC）ならびにヒト皮膚由来微小血管内皮細胞（HMVEC）両細胞に対し，明らかな増殖抑制作用がみられた。

4. タヒボ茶精製物ナフトキノンの“二重移植腫瘍系”での抗腫瘍効果

タヒボ茶抽出物中の有効成分であるナフトキノンを精製して“二重移植腫瘍系”で抗腫瘍効果を調べたところ，図5に示すように精製したナフトキノンにはほとんど抗腫瘍効果がみられなかった。

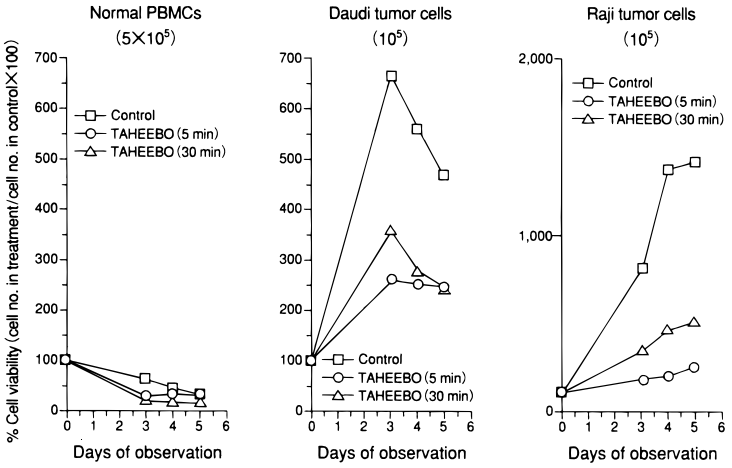

図 2 Direct *in vitro* cytotoxicity of TAHEEBO

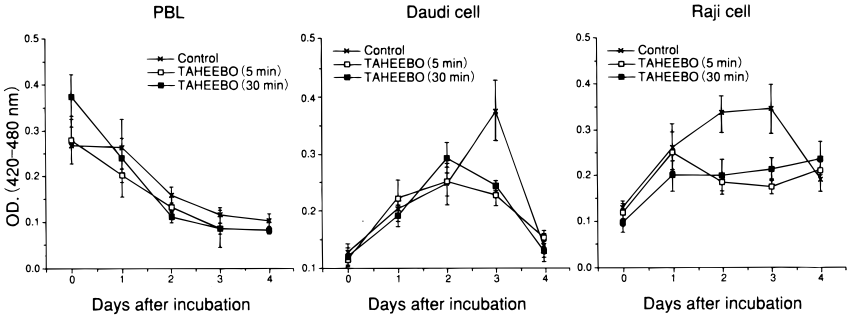

図 3 タヒボ抽出物の腫瘍細胞増殖抑制効果

III. 考　察

前報[7]にてタヒボ茶抽出物は“二重移植腫瘍系”で左・右の腫瘍の増殖を抑制し，血清IAPを誘導しマクロファージを活性化することを見いだした。さらに腫瘍細胞に直接働いて，腫瘍細胞の浸潤を阻害することを明らかにした。

今回，タヒボ茶抽出物は腫瘍細胞に対しアポトーシスを誘導し，正常リンパ球には誘導しないこと（図1），血管内皮細胞に対し増殖抑制作用を示すこと（図4）を明らかにしたが，今まで調べてきた他の生物製剤（PSK[4,6]，セファランチン[4,12]）と比較すると表1のようになり，少しずつ作用が異なることがわかった。

次に今まで調べてきた生物製剤（BRM）の“二重移植腫瘍系”における抗腫瘍効果と血清immunosuppresive acidic protein（IAP）測定によるマクロファージ活性化能を比較してみると表2のようになる。担子菌抽出物PSK，生薬セファランチン，樹木茶抽出物タヒボ茶は抗腫瘍効果もある

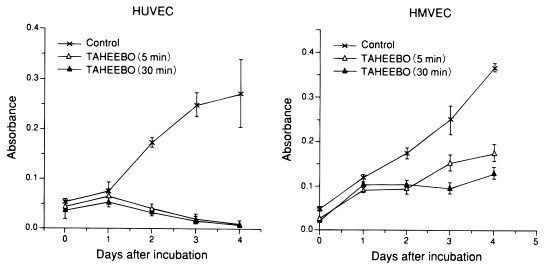

図 4 タヒボ抽出物の血管内皮細胞増殖抑制効果

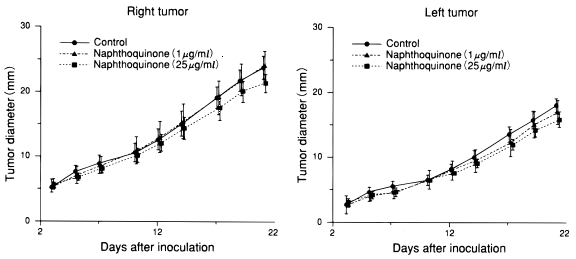

図 5 Antitumor effect of naphthoquinone

表 1 Comparison of biological activities among biological preparations

Biological preparations	Anti-metastatic effect in “double grafted tumor system”	Inhibition of tumor cell invasion	Induction of apoptosis		Growth inhibition of vascular endothelial cells	
			Tumor cell	PBL	HUVEC	HMVEC
PSK	++	+	±	−	−	−
Cepharanthin	+	+	++	±	+	±
TAHEEBO tea	+	+	+	−	+	+

し，マクロファージ活性化能があるのに対し，それぞれの精製物であるβ-グルカン，アルカロイド，ナフトキノンは抗腫瘍効果も弱く，マクロファージ活性化能も弱いことがわかった。すなわち抽出物のほうが精製物よりマクロファージにより認識されやすく，抗腫瘍免疫カスケード反応が起こり抗腫瘍効果を示すことがわかった。

従来の西洋医学が，化学構造が明確な精製されたものを薬剤として認め，漢方医学では数種類の生薬を混合したものを漢方薬として使っている。しかし精製物は量を間違えると毒物となり，副作用が常に付きまとい，医療事故が後を絶たない。一方，漢方薬は何が効いているかわからない欠点があり，他の西洋医薬との併用で副作用が起こる危険性がある。そこでわれわれは西洋医学でも漢方医学でもない統合医学（integrative medicine）を考案した[13]。統合医学でいう薬とはPSK，セファランチン，タヒボ茶抽出物のごとく，構造と機能が明確な有効成分を含む抽出物をいい，これら抽出物は最近機能性食品として認められ，副作用が弱いのが特徴である。

表 2 Anti-metastatic effect and macrophage activation of biological preparations

Biological preparation	Double grafted tumor system				IAP induction in serum ($\mu g/ml \pm SD$)
	Primary tumor		Metastatic tumor		
	Tumor free/ tested	Tumor weight (g±SD)	Tumor free/ tested	Tumor weight (g±SD)	
Extract of *Coliolus versicolor*, Protein bound β-glucan (PSK)	4/8	0.7±0.52	2/8	1.4±0.74	930±77
Purified β-glucan from *Lentinus edodes* (LNT)	0/9	5.8±1.0	0/9	3.7±0.6	189±42
Extract of *Stephania cepharantha* HAYATA (CR)	3/8	1.2±0.6	2/8	2.5±0.88	840±62
Purified alkaloid, cepharanthine	5/10	1.3±2.7	0/10	3.2±0.97	320±34
Extract of TAHEEBO tea	3/7	1.1±1.4	1/7	1.9±0.9	452±58
Purified naphthoquinone	0/6	3.7±0.8	0/6	1.6±0.2	174±22
Control, saline	0/8	5.3±0.72	0/8	3.1±0.5	133±18

SD : standard deviation

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