Inhibition of tumor-associated antigens secreted from cancer cell lines by *Taimatsu* fermented rice germ solution containing inositol hexaphosphate (IP6)

Nobunao IKEWAKI Toru ISHIZUKI* Masahiko NAKAMURA*
Ryutarou TAKAKI* Miki KANAI* Gengou HIGUCHI* Teruyoshi KONDO**

Abstract

Inositol hexaphosphate (IP6), a naturally polyphosphorylated carbohydrate, has been reported to have significant anticancer activity against numerous tumors, such as colon, prostate, breast, liver and rhabdomyosarcomas. Recently, we established a new IP6-containing solution, designated as T-IP6, produced by ***Taimatsu*** fermented rice germ. In this study, we examined the inhibitory effects of tumor-associated antigens secreted from a gastric cancer cell line (MKN-45) and a hepatocellular carcinoma cell line (HepG2) exposed to T-IP6. The tumor-associated antigens, such as carcinoembryonic antigen (CEA) and protein induced by vitamin K absence or antagonists-II (PIVKA-II), secreted from these cancer cell lines cultured with T-IP6 were significantly inhibited ($P<0.01$), compared with those in control cells (cultured without T-IP6) at 72 hrs, and this inhibition was accompanied by morphological changes, respectively. In addition, T-IP6 inhibited CEA and PIVKA-II in a dose-dependent manner. These findings suggest that T-IP6 has an immunoinhibitory and normalization effect in human cancer cells.

Key words : CEA, HepG2 cells, MKN-45 cells, PIVKA-II, T-IP6
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Introduction

Inositol hexaphosphate (IP6) is a polyphosphorylated carbohydrate that is ubiquitous in plants such as grains and legumes. IP6 is also found in most mammalian cells and is particularly ubiquitous in cell membranes, where it is found in conjugation with lipids as a phosphatidylinositol and plays a biologically significant role in intracellular signal transduction systems involved in cellular proliferation and differentiation.[1-3]

IP6 serves as a natural antioxidant and possibly as a neurotransmitter. In fact, the phosphate groupings at positions 1, 2, and 3 (axial-equatorial-axial) are unique for IP6, providing a specific interaction with iron that completely inhibits its ability to catalyze hydroxyl radical formation.[4] The antioxidant action of IP6 is widely recognized and accepted.

On the other hand, a novel anticancer action of IP6 has also been shown using both ***in vivo*** and ***in vitro*** experiments against various tumors, such as colon, prostate, breast, liver and rhabdomyosarcomas; this anticancer action occurs primarily via the regulation and suppression of cellular proliferation and cell growth.[3, 5-10] In addition, the inhibitory effects of vascular endothelial growth factor (VEGF) by IP6 have also been investigated.[11] VEGF is secreted by numerous tumor cells

Laboratory of Clinical Immunology, Department of Animal Pharmaceutical Science, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare
1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki, 882-8508, Japan
*Taimatsu Foods Corporation, 1345 Muramatsu, Gosenshi, Niigata 959-1794, Japan
**Department of Clinical Engineering, School of Health Science, Kyushu University of Health and Welfare
1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki, 882-8508, Japan

and promotes the proliferation of endothelial cells and the remodeling of neo-vessels. The induction of tumor vascularization is mediated by the release of the angiogenic factor VEGF. Thus, the inhibition of VEGF is thought to be the most specific means of confirming the anticancer action of IP6. However, the potential action of IP6 on the inhibition of tumor-associated antigens (tumor cell markers), such as carcinoembryonic antigen (CEA) [12] and protein induced by vitamin K absence or antagonists-II (PIVKA-II),[13] secreted by gastric cancer cells and hepatocellular carcinoma cells is poorly understood.

Recently, we succeeded in establishing a new solution, designated as T-IP6, containing IP6 produced by *Taimatsu* fermented rice germ; this solution is commercially available as a health food supplement.[14] In the present study, to confirm the ability of T-IP6 to act as an anticancer agent, we investigated the inhibitory effects of T-IP6 against CEA and PIVKA-II secreted from cancer cell lines using a gastric cancer cell line, MKN-45 and a hepatocellular carcinoma cell line, HepG2.

Materials and Methods

Reagents

The *Taimatsu* fermented rice germ solution containing IP6 (approximately 8.0 mg/mL), designated as T-IP6, was prepared by Taimatsu Foods Co. (Niigata, Japan) using the latest biological culturing and preparative techniques and is currently available commercially as a health food supplement.[14] A chemiluminescent enzyme immunoassay (CLEIA) kit for human carcinoembryonic antigen (CEA) was purchased from Sysmex Co. (Hyogo, Japan). An electro-chemiluminescence immunoassay (ECLIA) kit for human protein induced by vitamin K absence or antagonists-II (PIVKA-II) was purchased from Eisai Co. (Tokyo, Japan).

Cell lines

The gastric cancer cell line (MKN-45) and hepatocellular carcinoma cell line (HepG2) used in this study were supplied by the Japanese Research Resource Bank (JRRB) (Tokyo, Japan) and were cultured in RPMI 1640 medium (GIBCO) and advanced-DMEM medium (GIBCO) supplemented with 10 mM HEPES buffer, 2 mM glutamine, and 10% fetal calf serum (FCS) (GIBCO) (subsequently referred to as complete medium), respectively.

Preparation of MKN-45 and HepG2 cells cultured with T-IP6

The MKN-45 and HepG2 cells ($1x10^4$) in the complete medium described above were cultured in flasks (Sumitomo Co., Tokyo, Japan) with or without T-IP6 at various dilutions (1:100, 1:50, 1:25, or 1:10) at 37℃ for 72 hrs. Culture supernatants were used for the CEA and PIVKA-II assays. Morphological changes in the cells were observed using a phase-contrast microscope (x100), and photographs were taken using a camera body (Olympus Co., Tokyo, Japan) under the same conditions.

Assays for the detection of CEA and PIVKA-II in the culture supernatants

The detection of CEA and PIVKA-II in the culture supernatants obtained from the MKN-45 and HepG2 cells cultured with or without T-IP6 at various dilutions was performed using the CLEIA and ECLIA kits, respectively. The experiments were performed in triplicate and were repeated three times.

Statistical analysis

The mean and standard deviations (mean ± SD) were calculated. The statistical analysis was performed using the Student *t*-test or ANOVA (Dunnett test). Differences were considered significant when the *P* value was less than 0.05.

Results and Discussion

Inositol hexaphosphate (IP6) is present in most mammalian cell membranes, where it plays an important role in regulating vital cellular functions, such as cellular proliferation and differentiation, by participating in intracellular signal transduction.[1-3] In addition, IP6 has been reported to have some anticancer activities against numerous tumors, such as colon, prostate, breast, liver and rhabdomyosarcomas.[3, 5-10]

Recently, we succeeded in establishing a new solution, designated as T-IP6, containing IP6 produced by *Taimatsu* fermented rice germ; this solution is commercially available as a health food supplement,[14] with the

expectation that T-IP6 may have an anticancer action. In this study, to confirm the anticancer action of T-IP6, we investigated the inhibitory effects of T-IP6 against tumor-associated antigens (CEA and PIVKA-II) secreted from two types of cancer cell lines, MKN-45 (a gastric cancer cell line) and HepG2 (a hepatocellular carcinoma cell line). CEA is a biomarker that is present at high levels in serum from individuals with colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, lung carcinoma and breast carcinoma, but not in normal healthy adult individuals.[12] Meanwhile, PIVKA-II is an abnormal prothrombin identified in individuals with hepatocellular carcinoma (HCC) but not in normal healthy individuals that has been suggested to be a prognostic biomarker for rapid tumor progression.[13]

First, the inhibitions of CEA and PIVKA-II secreted from MKN-45 and HepG2 cells by T-IP6 (1:10) were analyzed using CLEIA and ECLIA systems, respectively. As shown in Fig. 1, the CEA secreted from MKN-45 cells cultured with T-IP6 was significantly inhibited (44.4 ng/mL, on average) ($P<0.01$) at 72 hrs, compared with that in control cells (120.3 ng/mL, on average) cultured without T-IP6. On the other hand, the PIVKA-II secreted from HepG2 cells cultured with T-IP6 was also significantly inhibited (123.9 mAU/mL, on average) ($P<0.01$) at 72 hrs, compared with control cells (449.7 mAU/mL, on average) cultured without T-IP6 (Fig. 2). These findings suggest that T-IP6 induced the differentiation of these cell lines from malignant phenotypes to normal phenotypes under the cell reversion system. Furthermore, these data strongly suggest that the T-IP6 action is involved in cell cycle regulatory genes, differentiation genes, and oncogenes mediated by various intracellular signal transduction pathways. In particular, tumor suppressor genes, such as p53, may be associated with the anticancer action of T-IP6. In fact, it has been reported that the inhibition of proliferation and growth in the human colon carcinoma cell line (HT-29) by IP6 is influenced by tumor suppressor genes (p53 and p21WAF1/CIP1).[15] With this point in mind, we suggested that T-IP6 also up-regulates the expressions of the p53 and p21WAF1/CIP1 genes, and their regulation by T-IP6 may induce the inhibition of CEA and PIVKA-II secreted from MKN-45 and HepG2 cells. Since the loss of p53 function enhances the resistance of cancer cells to chemotherapeutic agents, the T-IP6-induced stimulation of the p53 gene may serve as an attractive adjuvant for several chemotherapeutic agents.

In this study, the inhibition of CEA and PIVKA-II by T-IP6 in MNK-45 and HepG2 cells was accompanied by morphological changes (Fig. 3), suggesting that these cells decreased during cell proliferation and growth. Furthermore, these cells may have entered an apoptotic phase as a result of the modulation of cell surface antigen expression. For example, mucin marker (Gal-GalNAc) expressed by precancer and cancer cells of the colon, but not by normal cells, is reportedly modulated by IP-6 treatment, resulting in the high levels of Gal-GalNAc expression on these cells being decreased by the regulation of mucin synthesis, resulting in conversion to a normal cell phenotype.[16] Thus, we are vigorously investigating the reason for the T-IP6-induced morphological changes in NKM-45 and HepG2 cells using several apoptotic analysis techniques as a future step.

Next, the inhibitions of CEA and PIVKA-II by T-IP6 at various dilutions (1:100, 1:50. 1:25, or 1:10) in MKN-45 and HepG2 cells were also analyzed. Figures 4 and 5 showed that T-IP6 significantly inhibited ($P<0.05$) CEA and PIVKA-II secreted from these cancer cells. These data clearly indicate that the inhibition of CEA and PIVKA-II occurs in a dose-dependent manner.

The mechanisms involved in the inhibition of CEA and PIVKA-II by T-IP6 are not fully understood at present. However, the anticancer action of IP6 is based on the hypotheses that exogenously administered IP6 may be internalized, dephosphorylated to IP1-5, and that IP3 in particular may affect the anticancer action. In fact, IP3 is known to have important roles in cellular signal transduction, the regulation of cell function, and cell proliferation/differentiation in various cells.[17, 18] For example, when the concentration of IP3 in cancer cells is low, these cells grow vigorously without exhibiting regular cell growth cycle. In contrast, when the concentration of IP3 in cancer cells is high, these cells stop growing. Thus, these findings indicate that IP3 plays critical roles in cell proliferation/growth.

Two possible reasons can be considered explaining the T-IP6-induced inhibition of the secretion of CEA and

PIVKA-II from MKN-45 and HepG2 cells. First, the exogenous administration of T-IP6 to MKN-45 and HepG2 cells may result in its internalization and dephosphorylation to IP3, increasing the concentration of IP3 within the cells and effectively suppressing cell proliferation, thereby markedly inhibiting CEA and PIVKA-II secretion. Second, phospholipase C (PLC) within the cells may be activated by the administration of T-IP6, resulting in the dephosphorylation of phosphatidylinositol binding IP6 to IP3 and increasing the concentration of IP3 within the cells, thereby markedly inhibiting CEA and PIVKA-II secretion. In fact, a novel mechanism of IP6 against anticancer has been reported to depend on the activation of PLC.[19] However, the detailed inhibitory mechanisms of CEA and PIVKA-II by T-IP6 are not fully understood at the present time. Thus, we are now vigorously investigating how to analyze these mechanisms in our laboratories.

In conclusion, *Taimatsu* fermented rice germ solution containing IP6 (T-IP6) significantly inhibited the secretion of CEA and PIVKA-II from MKN-45 and HepG2 cells. These findings provide the firs evidence and also strongly indicate that T-IP6 has highly critical properties in anticancer actions. Further analyses are needed to demonstrate the detailed mechanisms of these findings at the cellular and molecular levels.

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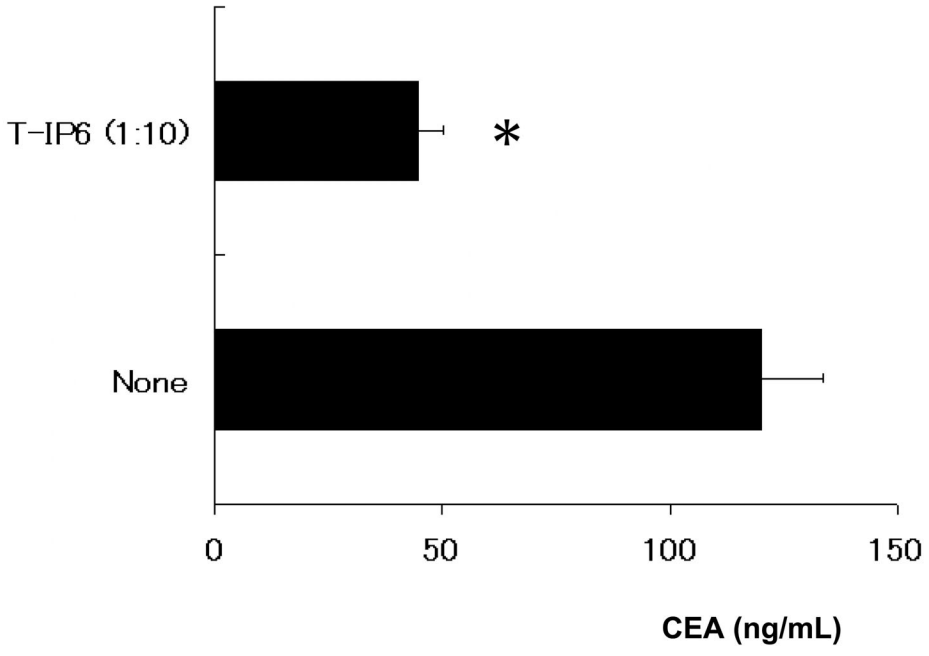

Figure 1. T-IP6-induced inhibition of CEA secreted from MKN-45 cells. MKN-45 cells were cultured with or without T-IP6 (1:10) for 72 hrs. The CEA in the culture supernatants was assayed using a CLEIA kit (see Materials and Methods section for details). The experiment was performed in triplicate and was repeated three times. The statistical analysis was performed using the Student *t*-test. *$P<0.01$, none vs. T-IP6.

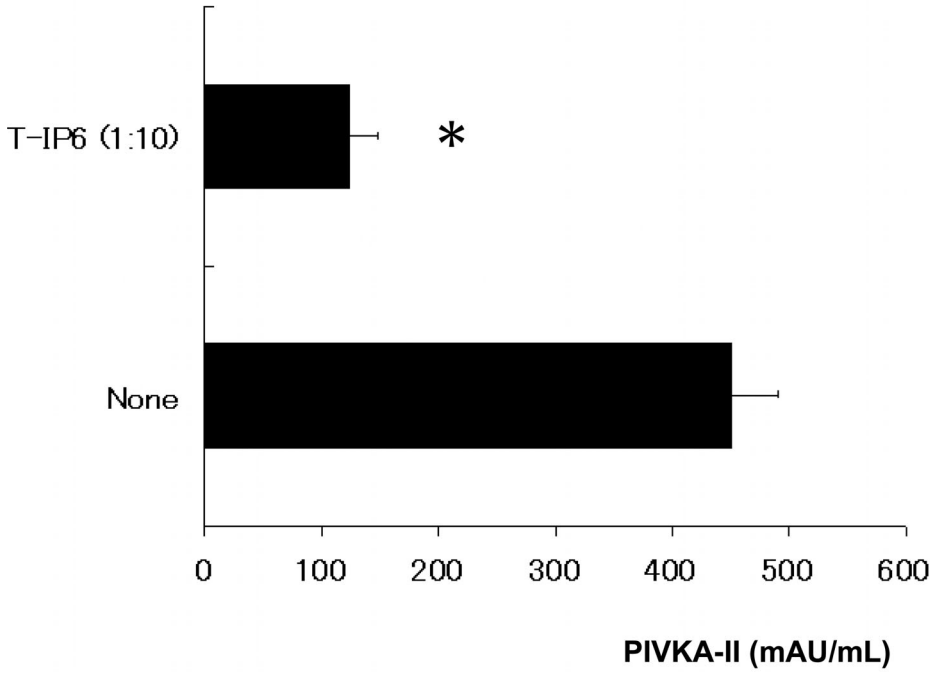

Figure 2. T-IP6-induced inhibition of PIVKA-II secreted from HepG2 cells. HepG2 cells were cultured with or without T-IP6 (1:10) for 72 hrs. The PIVKA-II in the culture supernatants was assayed using an ECLIA kit (see Materials and Methods section for details). The experiment was performed in triplicate and was repeated three times. The statistical analysis was performed using the Student *t*-test. $^{*}P<0.01$, none vs. T-IP6.

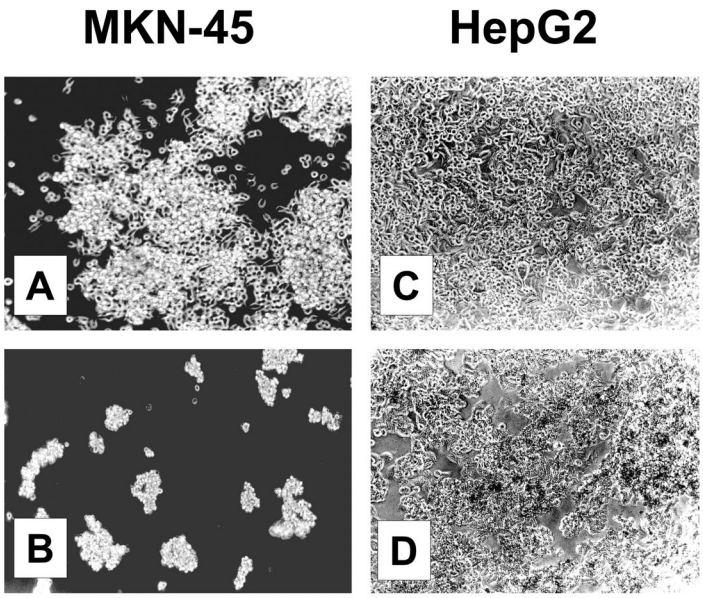

Figure 3. T-IP6-induced morphological changes in MKN-45 and HepG2 cells. MKN-45 and HepG2 cells were cultured with or without T-IP6 (1:10) for 72 hrs (see Materials and Methods section for details). The morphological changes were observed using phase-contrast microscopy (x100). Photographs were taken with an Olympus camera body under the same conditions. A and C: cultured without T-IP6. B and D: cultured with T-IP6.

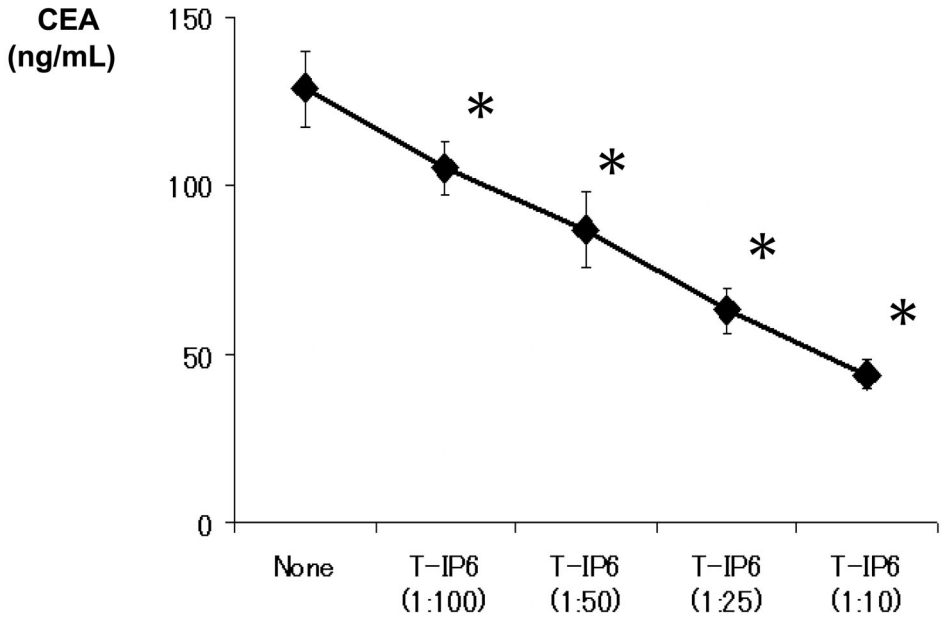

Figure 4. Dose-dependent inhibition of CEA secreted from MKN-45 cells cultured with T-IP6. MKN-45 cells were cultured with or without T-IP6 at various dilutions (1:00, 1:50, 1:25, or 1:10) for 72 hrs. The CEA in the culture supernatants was assayed using a CLEIA kit (see Materials and Methods section for details). The experiment was performed in triplicate and was repeated three times. The statistical analysis was performed using an ANOVA (Dunnett test). *$P<0.05$, none vs. T-IP6 (1:100, 1:50, 1:25, or 1:10, respectively).

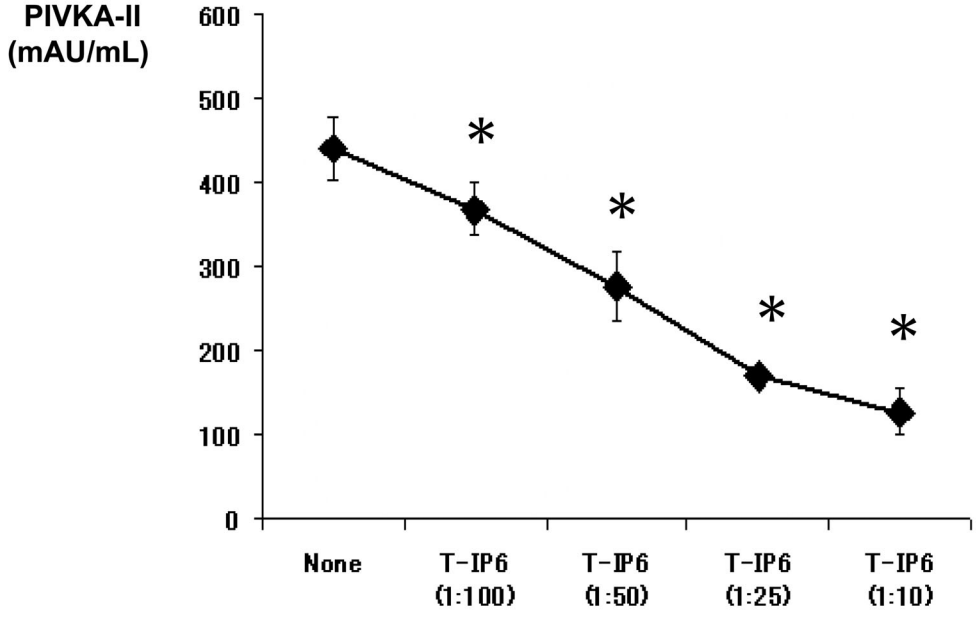

Figure 5. Dose-dependent inhibition of PIVKA-II secreted from HepG2 cells cultured with T-IP6. HepG2 cells were cultured with or without T-IP6 at various dilutions (1:00, 1:50, 1:25, or 1:10) for 72 hrs. The PIVKA-II in the culture supernatants was assayed using an ECLIA kit (see Materials and Methods section for details). The experiment was performed in triplicate and was repeated three times. The statistical analysis was performed using an ANOVA (Dunnett test). *$P<0.05$, none vs. T-IP6 (1:100, 1:50, 1:25, or 1:10, respectively).

IP6 含有たいまつ米胚芽発酵液による癌細胞株から分泌される腫瘍関連抗原の抑制

池脇　信直　　石附　亨*　　中村　雅彦*　　高木　隆太郎*
金井　美樹*　　樋口　元剛*　　近藤　照義**

九州保健福祉大学薬学部動物生命薬科学科　臨床免疫学研究室
〒882-8508　宮崎県延岡市吉野町1714-1
*たいまつ食品株式会社　〒959-1794　新潟県五泉市村松1345
**九州保健福祉大学保健科学部臨床工学科　〒882-8508　宮崎県延岡市吉野町1714-1

要旨

本研究では、我々が開発したIP6含有たいまつ米胚芽発酵液（T-IP6）の癌細胞株(MKN-45およびHepG2)から分泌される腫瘍関連抗原（CEAまたはPIVKA-II）に対する影響を化学発光および電気化学発光酵素免疫測定法で解析した。その結果、T-IP6で培養したMKN-45およびHepG2細胞から分泌されるCEAまたはPIVKA-IIは、細胞の形態変化を伴いながら有意に抑制された（$P<0.01$）。また、T-IP6によるCEAまたはPIVKA-IIの抑制作用は濃度依存性であった（$P<0.05$）。以上の結果から、T-IP6にはヒトの癌細胞を正常化させる作用があることがわかった。

キーワード：CEA　HepG2細胞　MKN-45細胞　PIVKA-II　T-IP6
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