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学位論文題目	Epicatechin-copper(Ⅱ) complexes: damage of small intestinal epithelium （エピカテキン－銅（Ⅱ）錯体：小腸細胞の損傷）
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論 文 内 容 要 旨

Abstract—Four epicatechins [(-)-epicatechin (EC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCg)] and their corresponding copper complexes were compared with regrad to their effect on the viability of Caco-2 colon cancer cell in vitro, measured by 3-(4,5-dimethylthyazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. The viability of Caco-2 cells exposed to EC (1 mM), ECg (1 mM) or EGC (1mM) respectively, for 30 min, was comparable to that of the saline control group, while EGCg (1 mM) apparently enhanced cellular activity. In contrast, the cells treated with epicatechin-copper complexes were killed. Bivalent copper (1 mM), in similar conditions, did not affect the cells. No cell leakage or other histological differences were observed. The suggested mechanism of killing is OH radical attack, produced in the presence of epicatechin-copper complexes, but not in the presence of either of the epicatechins or copper alone. The reaction sites are discussed.

Methods—*Cell line*: The American Type Culture Collection Caco-2 cell line from master stock was expanded in Petri dishes, using standard culture conditions. Confluent cell monolayers were harvested and dispersed within poly-L-Lysine coated 24-well culture plates, at a low density of 7×10^4 cells/mL

Incubation of cells with catechins and copper sulfate: The cells were washed in prewarmed saline, followed by 5 mM stock epicatechins aqueous solutions, to a final concentration of 1 mM in saline. The copper complexes were formed by subsequently adding copper sulfate 10 mM to a final concentration of 1 mM/well. The controls were incubated in saline alone.

The final experimental incubation time of 30 minutes was chosen after calibration experiments were run from 15 min up to 4 h. Following incubation, the reaction medium was removed, the cells were washed in saline, then submitted to the MTT assay.

MTT Assay principle: the viable cell number/well is directly proportional to the production of formazan which, following solubilization, is measured spectrophotometrically(UV).

Statistics: Experiments were performed in triplicate (±SD). Statistical comparisons were made by ANOVA followed by an unpaired (homoscedastic) two-tailed Student's t-test.

Results－We clearly demonstrated that, compared to the control group, neither the four epicatechins (1 mM) nor copper (1 mM) produce a decrease in the viability of Caco-2 cells (t-test, $P_{control\text{-}copper} = 0.025$, $P_{control\text{-}EC} = 0.971$). In contrast, the presence of any of the four epicatechin-copper complexes (1 mM) considerably decreases the cells' viability, as demons

trated by the significant drop in the production of formazan by all the complex-treated cells (ANOVA, $P_{four\ complexes} = 0.39$; t-test, $P_{control\text{-}complex} < 0.001$).
Although it has been reported that 1mM epicatechins induce cell death in our study the viability of cells treated with 1mM of EC, ECg, and EGC, respectively, is similar to the control (t-test, $P_{control\text{-}EC} = 0.97$, $P_{control\text{-}ECg} = 0.024$, $P_{control\text{-}EGC} = 0.088$; or ANOVA, $p_{control.\ EC.\ ECg.\ EGC} = 0.041$). Moreover, the activity of those cells treated with EGCg is significantly increased from the control (t-test, $P_{control\text{-}EGCg} < 0.001$).

From a morphological point of view it is also clearly visible on the photos taken under the microscope that the cells treated with the epicatechin-copper complexes remain unstained, while the control and those treated only with epicatechins or copper alone appear stained (see article Fig. 5). Therefore the latter are active. Moreover, from the general aspect of the cells under microscope, it can be inferred that the death is relatively sudden, and the loss of dehydrogenase activity precedes the membrane permeabilization, since no apparent morphological changes (cell swelling/shrinkage or debris) are visible.
Discussion—MTT's intracellular conversion into formazans depends on the NADH and NADPH dehydrogenases. Thus, it is an indicator of cellular metabolic activity. However, the MTT assay as an estimate of cell viability actually measures endocytosis and exocytosis, a fundamental feature of most living cells. It has been reported that flavonoids can inhibit MTT-formazan exocytosis, therefor our results showing slightly more formazan in the EGCg treated group, compared to the control group, does not necessarily imply that EGCg has induced enzymatic changes at the NADH/NADPH level. The augmented values of formazan read could be due to a possible specific mechanism of exocytosis inhibition.

Mechanisms of flavonoid action; Epigallocatechin gallate significantly enhanced the activity of Caco-2 cells, while epicatechin, epicatechin-gallate and epigallocatechin did not show boosting effects. On the other hand all four epicatechin-copper complexes studied induced cell death.

The metal-chelating property of catechins was, until recently, thought to be responsiblefor the anti-oxidant effects of these compounds in vivo. However, the autooxidation of catechins produces H_2O_2, the process being catalyzed by copper. Other studies also showed that, in the presence of copper, epicatechins produce hydroxyl radicals, enhancing lipid peroxidation and/or killing bacteria. The mechanism proposed involves molecular oxygenthat interacts with the complex, near the cell membrane, to produce free radicals, which are toxic for the cells. It was likely that this was the mechanism by which the intestinalcells in our study were killed, too.

However, we observed for the first time that a mechanism by which epicatechins deliver copper inside the cell, where the metal initiates oxidation, couldn't be ruled out. (Recently, our group has proved this mechanism of transport; *article in journal reviews.*)

学 位 論 文 審 査 の 結 果 の 要 旨

近年、緑茶カテキンの抗酸化性が注目され、酸化ストレスに対する多くの研究が活発に行われている。一方、銅イオンの存在下にはカテキン類が活性酸素種を産生し、DNA鎖の切断や殺菌作用を示すことが報告されている。

申請者は、緑茶を飲用したときカテキン類が代謝を受ける前に最初に遭遇するであろう消化管細胞への作用に着目した。小腸培養ガン細胞Caco-2に対するエピカテキン－銅錯体の作用を検討し、そのメカニズムについて報告した。細胞活性の測定には3-(4, 5-dimethylthyazole-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay)法を用いている。

その結果、細胞は形態学的には破壊されていないにも関わらず、エピカテキン－銅錯体によって瞬時に死滅することを明らかにした。そして、細胞死のメカニズムは、エピカテキン－銅錯体によって生成するヒドロキシルラジカルなど活性酸素種の攻撃によるとしている。

本論文は、エピカテキン－銅錯体が細胞死を引き起こすことを示し、ガン細胞破壊への応用の可能性を示しており、博士（医学）の学位授与に値するものと評価された。