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EPICATECHINS INDUCE DEATH OF CACO-2 COLON CANCER CELLS BY RAISING THE INTRACELLULAR LEVEL OF COPPER (II)

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ABSTRACT

We have recently shown that green tea epicatechins complexed with copper kill colon cancer cells of the Caco-2 line, in vitro, at concentrations for which copper alone does not affect cell viability. Attempting to elucidate the mechanism of killing, we found hereafter that epicatechins actively transport copper inside the cells promoting a prooxidant state eventually leading to apoptosis.

Key words: epigallocatechin, angiogenesis, copper, active transport, Caco-2.

1. Introduction

In the progression phase of cancer, in order to expand beyond a tiny cluster, tumor cells need to accumulate copper, an essential element for growth factors involved in the formation of new blood vessels. Clinical studies already show that tetrathiomolybdate (TM) arrests cancer growth by strongly binding copper, depriving tumors of their own supply [1]. The idea of targeting copper as a "common denominator" of angiogenesis is one of the most recent breakthroughs in the fight against cancer.

On the other hand, the generation of reactive oxygen species (ROS) is one of the main mechanisms by which normal cells are fighting their tumorous counterparts, and copper ions are strong oxidants that can produce ROS. A chelator selectively delivering copper to a cancerous cluster of cells would promote a prooxidant state, eventually leading to apoptosis [2, 3].

Epicatechins, the major polyphenol components of green tea, are able to chelate copper. They have been described as inhibitors of cancer growth [4-7] as well as of angiogenesis [8, 9]. However, due to their molecular structure, epicatechins bound copper only loosely compared to TM, so it's unlikely that their mechanisms of action are similar. Moreover, while epicatechins are well known antioxidants, epicatechin-copper complexes are strong oxidants [10-13]. We have already shown that, at concentrations where copper ions alone cannot kill cells, their corresponding epicatechin-copper complexes are able to induce cell death [14].

Two explanations were possible: either the complexes attacked the cell membrane by producing extracellular ROS, or they transported copper through the cell membrane and initiated oxidation in-

Abbreviations: EC, epicatechin; ECg, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; MTT, 3-(4,5dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

tracellulary. The present study argues in favor of the later mechanism.

The highest local concentration of green tea polyphenols is found in the gut lumen [15], and they may have a direct impact on the gut mucosa. Using an MTT assay for cell viability and a rhodanine stain as indicator for the level of intracellular copper, we checked the ability of the epicatechin－copper complexes to initiate apoptosis within Caco-2 cells of the colon cancer line.

2. Materials & Methods

2.1. Cell line and chemicals

The American Type Culture Collection Caco-2 cell line from master stock (generations 40-44) was cultured on microscope cover glass in 24 well dishes under standard conditions, as previously described [14].

Stock aqueous solutions of 5mM epicatechins (Funakoshi, Japan) and 10mM copper sulfate (Nakarai Chemicals Ltd.) were made using sterile purified water (Mitsu-Ebitsu, Japan).

Stock ethanol solution of 0.1% 5-(4-dimethylaminobenzylidene)-2-thioxo-4-thiazolidinone (rhodanine, Wako, Japan)

2.2. Incubation of cells with catechins and copper sulfate

The experiments were designed at 1mM aqueous solutions of epicatechins, close to a physiological achievable concentration in the gut, as derived from the model standard infusion of tea [16, 17]. Seven days after seeding in 24 well plates (7 x 10^4cells/ well), the cells were washed 3 X 1mL prewarmed saline (37℃) and the necessary volume of saline was added, followed by 5mM stock epicatechins aqueous solutions, to a final concentration of 1mM. The copper complexes were formed by subsequently adding copper sulfate 10mM to a final concentration of 1mM/well. Other final concentrations of reagents were obtained in the same manner. The final volume of each well was 1mL in all experiments. The controls were incubated in saline alone. Incubation time was 30 min.

2.3 Rhodanine stain and viability assay.

After incubation with complexes, the cells were washed 3 X saline and rhodanine, which chelates copper, was used as an indicator of the cellular retained metal. The formalin-fixed red rhodanine-stained cells [18] were mounted on slides, scanned, and the intensity of the color was detected using UTHSCSA Image Tool software. Cell viability was determined using an MTT-assay, as previously described [14].

3. Results

Caco-2 cells incubated for 30 min in saline with four equimolar epicatechin-copper complexes, gave clear-positive red stain with rhodanine [18] as compared to non-complexed copper treated cells (Fig. 1). The amount of metal transported varies with the concentration of each complex, showing saturation at about 1mM complex for all four epicatechins investigated (Fig. 2). The red color intensity was maintained even after the cells were washed three times in ethanol, prompting us to infer that the copper was bound intracellulary. The cells treated with any of the four epicatechins alone, as well as

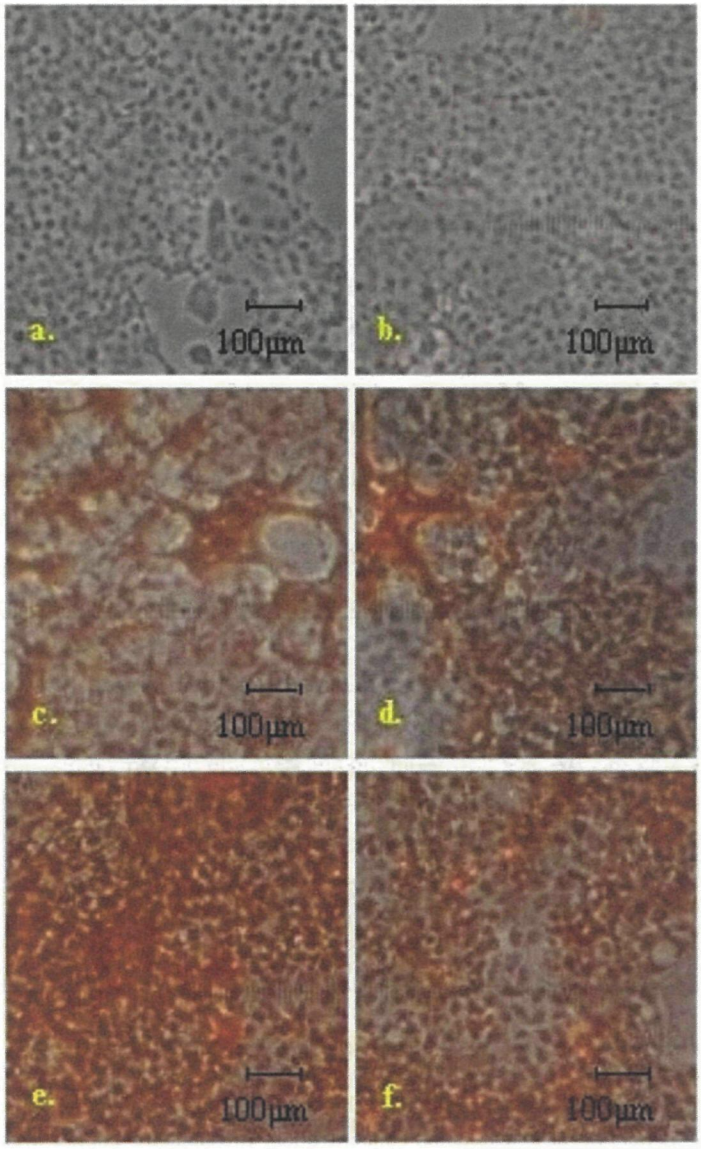

Figure 1. Rhodanine stain of Caco-2 cells treated with epicatechin and copper, compared to control and copper alone; a. Control; b. Copper; c. EC and copper; d. ECg and copper; e. EGC and copper; f. EGCg and copper; (magnification X 100).

the control incubated in saline only, did not show positive for the rhodanine stain. Treatment with 1 mM copper alone for 30 min in saline, did not lead to rhodanine detectable copper accumulation inside the cells, weather or not cells were preincubated with epicatechins.

The toxicity of test components was assessed by the cell uptake of MTT [14]. The viability of cells treated with 0.1mM complex is comparable to that of the control group, while cells treated with 1 mM or more complex were all killed.

Since the quantity of copper bound, as well as the type of binding may depend on the amount of

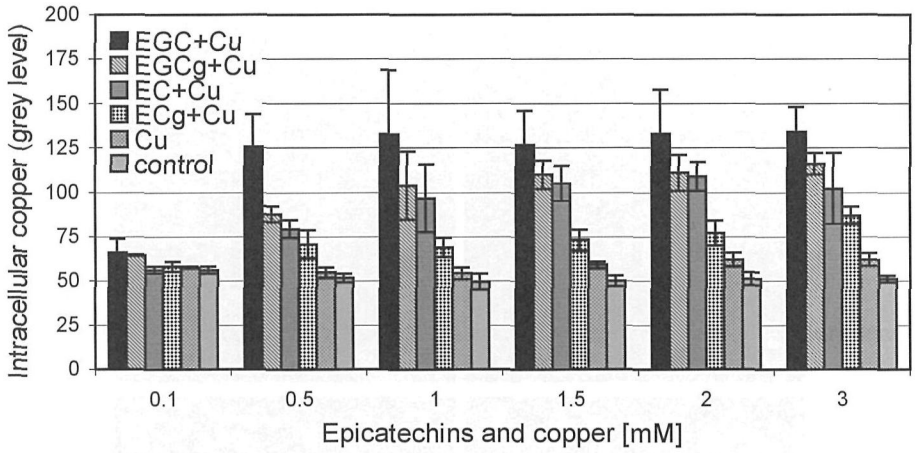

Figure 2. Copper transported inside the cells by four epicatechin-copper complexes, as a function of complex concentration (ECs : Cu molar ratio is 1:1). Intracellular copper was determined by color intensity with rhodanine.

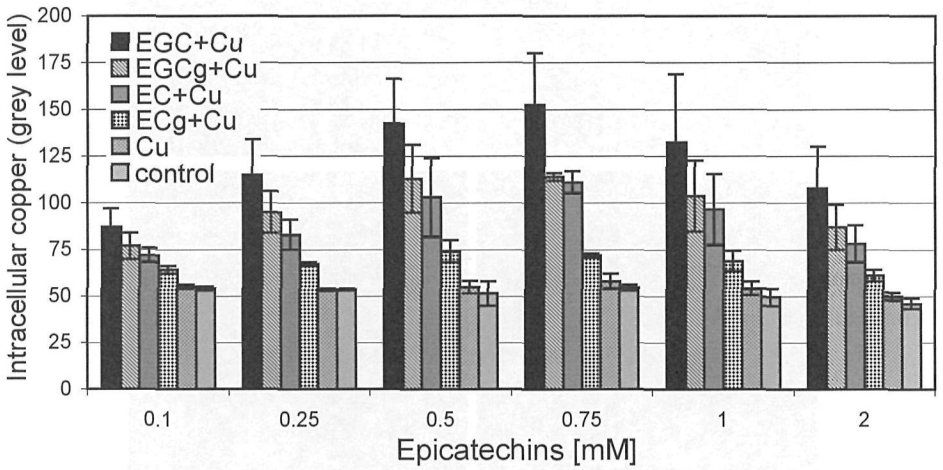

Figure 3. Copper (1mM) transported inside the cells by four epicatechin-copper complexes, as a function of epicatechins concentration. Intracellular copper was determined by color intensity with rhodanine.

epicatechins available [19], we also monitored the amount of copper transported as a function of the quantity of epicatechins present (Fig. 3). The transport seems most effective at epicatechin : copper ratios of 3:4 (0.75mM epicatechins : 1mM copper sulfate). It is possible that at higher ratios the antioxidant effect of epicatechins alone is not negligible.

4. Discussion

4.1. Active transport

The gap junctions are membrane channels that permit the transfer of small water-soluble molecules from the cytoplasm of one cell to that of its neighbors. Epicatechins are known to open the gap

junctional intercellular communication (GJIC) [20-24], which, in turn, also enhances the effects of other agents. However, preincubation with epicatechins (30 min), followed by washing with saline and addition of copper, did not result in the transport of this metal into the cytoplasm, prompting us to conclude that copper enters the cell only bound to epicatechins, in a complexed form.

On the other hand, the family of multidrug resistance protein receptors (MRP) [25] - and in particular MRP2 (also known as cMOAT, canalicular multispecific organic anion transporter) which is expressed on the apical membrane of the Caco-2 cells [26] - is involved in the epicatechin transport from the cytoplasm to the exterior of the cell. It is highly likely that the epicatechin-copper complexes are handled by the same receptors. In our experiments a concentration of 0.1mM complex delivers only a little amount of copper inside the cells (Fig. 2), not enough for killing them in 30 min. One explanation could be that, at a concentration of up to 0.1mM the MRP2 transporter is effectively efluxing the complex, while, above this concentration, it cannot cope with the transport and the metallic ion starts accumulating in the cell.

The fact that a concentration of 1mM complex is enough to kill the cells in 30 min [14], but further increasing the complex concentration does not increase the amount of copper delivered to the cytoplasm (Fig. 2), indicates that when all the cells are killed the transport stops, also supporting an active mechanism of transport.

Moreover, in order to establish that the intracellular copper detected is not due to passive osmosis (after cell death) through the membrane pores allegedly formed as a consequence of the attack of ROS generated extracellulary by the epicatechin-copper complexes, we ran an experiment where the cells were first treated with H_2O_2, 0.03%. This concentration mimics the amount of OH radicals generated by the 1mM complex [12] and it completely killed the cells as showed by the MTT viability test [14]. The cells thus treated, followed by epicatechin-copper incubation, showed no transport of copper.

Nevertheless, we cannot rule out a concerted mechanism, in which the transport is a consequence of dynamic local membrane permeabilization by the ROS generated extracellulary from the epicatechin-copper complex (oxidative stress permeabilization [27, 28]), followed by copper delivery into the cytoplasm. EGC was found most efficient in transporting copper inside the cell at all tested concentrations (Fig. 2 and Fig. 3). The second in order was EGCg, closely followed by EC. ECg transported only a very little amount of copper. The correlation between, on one hand, the order of efficiency in copper transportation (EGC >> EGCg > EC >> ECg) and, on the other hand, the finding that EGC in the presence of copper (30 min), produces significantly more radicals than either EGCg, EC, or ECg (unpublished data), suggests the above mentioned concerted mechanism.

Interestingly, epicatechin-copper complexes cleave isolated DNA (in vitro), with exactly the same order of activity [29]. One step further, our finding of a similar order for these compounds' capacity to deliver copper into the cytoplasm, definitely places epigallocatechin-copper complex as the most suitable candidate for oxidative damage in this series.

Finally, it is known that reducing GSH (γ-L-glutamyl-L-cysteinylglycine) concentration in tumor cells sensitizes them to a variety of treatments [30]. Also, cells transfected with an MRP1 cDNA construct are rapidly depleted of GSH when transferred from rich growth media to buffered saline, suggesting that they must be producing GSH at high rate in growth medium in order to counteract this drain.

There has not been found a simple way to stop this efflux or to compensate it with glutathione esters [25]. (MRP1 and MRP2 are high/close homologues). As we ran our experiments in saline, an over sensitization of Caco-2 cells to the epicatechin-copper complex due to depletion of GSH may have influenced the quantitative aspect of the copper transport mechanism described.

In conclusion, we have shown for the first time that, epicatechin-copper complexes have the ability to actively deliver copper across cell membranes, initiating intracellular oxidation. Despite a free copper occurrence of less than one atom per normal growing cell [31], under certain conditions of cellular oxidative stress copper is released [32], and epicatechins, present in human plasma in various concentrations [33, 34], may scavenge this metal at different sites [35]. Tumor cells, already more sensitive to the actions of epicatechins [5] and eager to acquire copper for angiogenesis (growth), could be more susceptible to the oxidative action of epicatechin-copper complexes than their normal counterparts.

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