

Molecular Mechanisms for Cancer Prevention by Phytonutrients: Posttranslational Modifications of Nrf2/ARE Transcription System Components.

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Introduction: Diet rich in fruit and vegetables have been recommended for reducing cancer risk. One mechanism for cancer prevention by dietary compounds is induction of phase II detoxification enzymes. Expression of phase II enzymes is regulated by the antioxidant response element (ARE) and the transcription factor Nrf2. Dietary inducers of Nrf2 such as polyphenols, isothiocyanate and carotenoid derivatives, activate ARE mainly through interactions with Keap1, a repressor of Nrf2. In addition, posttranslational modifications such as phosphorylation and acetylation, on Nrf2 or other proteins in this transcription system, might be involved in the activation by phytonutrients. Studies have shown that Casein kinase 2 (CK2) is part of the Nrf2/ARE regulation. In addition, previous studies have shown that the polyphenol and ARE inducer, curcumin, inhibits the histone acetyl transferase p300, whereas the polyphenol resveratrol activates the histone-deacetylase (HDAC) SIRT1, a member of the sirtuins class III family of HDACs that are NAD⁺-dependent. We aim to examine whether posttranslational modifications are involved in ARE/Nrf2 transcription system activation by various dietary compounds.

Results: Various phytonutrients such as curcumin and sulforaphane activated the Nrf2/ARE transcription system, resulting in Nrf2 accumulation and phase II enzymes induction. Human Nrf2 has a predicted MW of 66 kDa, but is detected by Western blotting at 98 and 118 kDa, which may correspond to modified states of Nrf2. Treatment with CK2 and MEK1/2 inhibitors reduced ARE transactivation and Nrf2 levels, under stimulation by phytonutrients. In contrast, p38 and JNK inhibitors did not have a significant effect. Furthermore, ARE transactivation by dietary compounds was inhibited by Nicotinamide, a physiological HDAC class III inhibitor. Inhibition was also detected with Trichostatin A, a potent HDAC class I and II inhibitor. Moreover, over-expression of p300 inhibited ARE activation by various phytonutrients. Thus, increased acetylation by all three reagents inhibits ARE activity.

Conclusions: We suggest that phosphorylation by CK2 and the MEK1/2 pathway is essential for ARE activation by dietary compounds, whereas other MAPK pathways, such as p38 and JNK, are apparently not involved in ARE/Nrf2 activation. Since histone acetylation increases transcriptional activity, the inhibition of ARE by increased acetylation suggests that this system is regulated by changes in the acetylation state of the relevant non-histone proteins. The study of the acetylated state of these proteins is in progress. The additional regulation by acetylation might explain synergistic activation of ARE by combinations of various dietary compounds.

THE RAPAMYCIN DERIVATIVE RAD001 (EVEROLIMUS) INHIBITS CELL PROLIFERATION AND INTERACTS WITH THE AKT-mTOR-p70S6K PATHWAY IN HUMAN MEDULLARY THYROID CARCINOMA CELLS.

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Introduction: Background: The effects of the rapamycin derivative RAD001 (everolimus) on neuroendocrine tumor cell proliferation, cell viability and hormone secretion are largely unknown. Over-expression of the proto-oncogene Akt/PKB has been demonstrated in some neuroendocrine tumor models: Akt may activate downstream proteins such as mTOR and p70S6K, inducing tumor cell proliferation. We have therefore explored the mechanism of action of RAD001 on cell proliferation and viability, and on Akt/mTOR/p70S6K pathway activation, in a human medullary thyroid carcinoma (MTC) cell line (TT cell line) and in cells derived from human MTCs. Aims: To evaluate the in vitro effects of RAD001 on human MTC cell line, TT, and on human MTC primary cultures.

Methods: Viability assays (XTT) and cell counting were used to determine the effects of RAD001 at different concentrations on tumor cell viability. Western blotting was used to characterize and analyze the effect of the drug on the expression of phosphorylated-Akt, phosphorylated-mTOR, and phosphorylated-p70S6K. Flow cytometry was performed to assess cell cycle arrest and apoptosis. IRMA determined RAD001 effect on calcitonin secretion from tumor cells.

Results: Treatment with RAD001 significantly inhibited cell viability in a dose- and time-dependent fashion and diminished phosphorylation of Akt downstream targets, mTOR and p70S6K, in both TT cell-line and cultured human MTCs. Akt activity was not affected by RAD001. RAD001 induced cell-cycle arrest in the G1/S phase, while having no significant apoptotic effect in TT cells.

Conclusions: In human MTC cell cultures, RAD001 significantly inhibited cell proliferation and the Akt-mTOR-p70S6 kinase signaling pathway downstream of Akt, in parallel with inducing cell-cycle arrest. RAD001 seems to have potent anti-tumoral effects in human MTC cells, which suggest that clinical trials of this agent are of considerable interest.

The peptide-hormone glucagon-like peptide 1 (GLP-1) activates AMP kinase and inhibits growth of breast cancer cells in vitro and in vivo.

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Introduction: GLP-1 is a peptide-hormone, secreted from endocrine L cells in the gut in response to food intake and modulates metabolic pathways. It activates a specific G-protein coupled receptor and promotes pancreatic β -cell proliferation and differentiation, increase insulin secretion and sensitivity and reduces appetite. Its levels are reduced in obesity and type 2 diabetic mellitus. GLP-1 has been suggested as a potential therapy for diabetes. As it is rapidly inactivated in the serum and its half-life is only 2 minutes, more stable GLP-1 receptor agonists have been developed. Exendin4 (Ex4) is a stable and potent GLP-1 receptor agonist, which has recently been approved by the FDA for the treatment of diabetes. As insulin resistance is associated with breast cancer, and as GLP-1 induces insulin sensitivity and is a differentiation factor, we hypothesized that it may affect growth of breast cancer cells in vivo and in vitro.

Results: Short-term effects of Ex4 on proliferation of breast cancer cells were tested using MTT assays and long-term effects were examined by colony formation assays. Treatment with Ex4 (5 nM, 48 hrs) reduced viability of the hormone receptor-positive MCF-7 and the hormone receptor-negative MDA-MB 231 breast cancer cells by 50%, but did not affect viability of human primary liver cells or the non-cancerous human embryonic kidney (HEK)-293 cells. Similarly, Ex4 (5 nM, 2 weeks) inhibited colony formation of the breast cancer cells but not of the control cells. Ex4 treatment increased apoptosis of the breast cancer cells, as indicated by annexin-V staining, and was associated with elevation of the tumor suppressors p53 and p21 protein levels. In order to assess in vivo activity, MDA-MB-231 cells were injected to both flanks of athymic mice, and pumps, which continuously release either Ex4 500ng/day, Ex4 2 μ g/day or a control vehicle were implanted subcutaneously. Ex4 inhibited tumor growth but did not affect weight or general health of the mice. Downstream signaling pathways that modulate GLP-1 activity were also evaluated. The classic GLP-1 receptor was not detected in breast cancer cells, but treatment with either Ex4 or GLP-1 elevated cAMP levels, and this elevation was blocked by GLP-1 receptor antagonist (exendin9-39), suggesting the existence of a non-classical GLP-1 receptor in breast cancer cells. GLP-1 and Ex4 treatment induced p38 MAPK and CREB phosphorylation and activated AMP kinase, and its down-stream target acetyl-CoA carboxylase. GLP-1 and Ex4 did not affect the ERK1/2 or AKT pathways.

Conclusions: These data indicate, for the first time, the endogenous hormone GLP-1 as an inhibitor of breast cancer cell growth. Reduced GLP-1 levels are, therefore, a potential novel link between diabetes and obesity and breast cancer. Importantly, as GLP-1 analogs are already being used in the clinic, their implementation for the prevention or treatment of breast cancer may be feasible

Glucagonoma and the glucagonoma syndrome-Cumulative experience and clinical characteristics of six patients treated in our facility between 1985 and 2008

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Introduction: Glucagonoma is a very rare pancreatic neuroendocrine tumor that usually arises from alpha cells in the tail of the pancreas and often is accompanied by a characteristic clinical syndrome.

Patients/ Methods: In this report we present the cumulative experience and the clinical characteristics of six patients diagnosed with glucagonoma and the glucagonoma syndrome and treated in our facility between 1985 and 2008.

Results: Although disease progression was highly variable amongst our patients, some features were similar. The average age at diagnosis was 54.5 years, the time from the onset of probable tumor related symptoms to diagnosis was prolonged: 39.6 months (ranging from 4 to 72months) before. The most common presenting symptoms were: weight loss 5/6 (83%), necrotizing migratory erythema (NME) 5/6 (83%), diabetes mellitus 4/6 (66.7%), diarrhea, weakness and thrombosis- 2/6 (33%), cheilosis or stomatitis, anemia and abdominal pain 1/6 (16.7%). Plasma glucagon level at diagnosis was 4835 pmol/L (ranging from over 10000 to 306 (N<50pmol/L)). The primary tumor was localizable by CT in 4/6 patients. Metastatic disease developed at some time during the course of the disease in all our patients with 4/6 presenting initially with hepatic metastasis. All patients were treated shortly after diagnosis and responded clinically to various analogues of somatostatin. 4/6 patients were treated with amino acid solutions, with an invariably good clinical response with respect to NME resolution. Surgical debulking was carried out in 3/6 patients. Peptide receptor radio-ligand therapy (PRRT) with 90Y-DOTATOC was usually carried out after failure of somatostatin analogues. Of the three patients receiving PRRT, two had significant clinical and radiological responses. Systemic chemotherapy was given to two patients (using combinations of streptozotocin, 5-FU, leucovorin, adriamycin and dacarbazine), usually leading to prolonged remissions. 4/6 patients died of the disease. Death occurred in average 4.25 (range 2-11) years after diagnosis and 6 (range 4-16) years after the initial clinical manifestations. The two surviving patients have been followed thus far for one and 7.5 years.

Conclusions: Our data indicate that a multimodal treatment approach with surgery, somatostatin analogues, PRRT, amino acid infusions and chemotherapy appear to improve patient quality of life and perhaps also affects patient survival.

Progression to advanced stage disease in a cellular model of prostate cancer is associated with methylation of the androgen receptor (AR) gene and transcriptional suppression of the insulin-like growth factor-I receptor (IGF-IR) gene

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Introduction: The progression of prostate cancer from an organ-confined, androgen-sensitive disease to a metastatic one is associated with dysregulation of androgen receptor (AR)-regulated target genes and with a decrease in insulin-like growth factor-I receptor (IGF-IR) expression. The molecular mechanisms that are responsible for regulation of the IGF-IR gene in prostate cancer, however, remain largely unidentified. Methylation of CpG islands is an epigenetic mechanism associated with gene silencing. Recent studies have demonstrated that methylation occurs early in prostate carcinogenesis and, furthermore, may contribute to androgen independence.

Methods: The aim of this study was to evaluate whether the low IGF-IR levels seen at metastatic prostate cancer stages are caused by direct methylation of the IGF-IR promoter or, indirectly, by dysregulated expression following methylation of an upstream regulator, including the AR promoter.

Results: Prostate cancer cell lines were treated with the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza), after which IGF-IR and AR levels were measured by Western blots and Real time-PCR. Results obtained showed no change in IGF-IR protein levels in 5-Aza-treated cells in comparison to untreated cells, suggesting that the IGF-IR gene is unmethylated in all cell lines tested. These results were confirmed using the sodium bisulfite-DNA sequencing method. Further analyses, however, demonstrated that the AR gene is hypermethylated in metastatic M12 and DU145 cells, but not in benign P69, cells. In addition, we showed that 5-Aza treatment, which caused demethylation of the AR promoter, led to a significant increase in the levels of IGF-IR mRNA, whereas addition of the AR inhibitor flutamide decreased the IGF-IR mRNA levels to basal values measured prior to the 5-Aza treatment. In addition we tested the methylation pattern of other genes involved in IGF-IR gene regulation using both 5-Aza treatment and sodium bisulfite analysis method. We didn't find methylation in the promoter regions of the BRCA1, KLF6, and estrogen receptor (ER) α genes. In contrast, we showed that the progesterone receptor (PR)-A and PR-B promoters are methylated in all cell lines tested.

Conclusions: Given that the IGF-IR gene has been identified as a target for AR action, our data is consistent with a model in which the AR gene undergoes methylation during progression of the disease, leading to dysregulation of AR targets, including the IGF-IR gene, at advanced stages. Our results provide evidence for the involvement of epigenetic mechanisms in prostate cancer progression.

Klotho: a tumor suppressor and modulator of the IGF-I pathway in human pancreatic cancer

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Introduction: Klotho is an anti-aging transmembrane protein, expressed in the brain, kidney and in various endocrine-related tissues including the pancreas. We have recently identified klotho as a tumor suppressor and a modulator of the insulin-like growth factor (IGF)-1 and the fibroblast growth factor (FGF) pathways in breast cancer. Pancreatic adenocarcinoma is one of the most lethal of human cancers. The IGF-1 pathway plays an important role in the pathogenesis of pancreatic cancer, IGF-I and IGF-I receptor (IGF-IR) are overexpressed in these tumors, and IGF-1R inhibition suppresses tumorigenicity in vitro and in vivo. Thus, the IGF pathway serves as a target for novel therapies against pancreatic cancer. As klotho inhibits the IGF-I pathway, we aimed to study its expression and activity in pancreatic cancer.

Results: Immunohistochemistry was used to analyze klotho expression in pancreatic tissues and revealed high klotho expression in normal pancreas, but only in 15% of pancreatic cancer samples. In pancreatic cancer cell lines klotho mRNA expression was 2000-times lower than in normal pancreas. Overexpression of klotho reduced colony formation of Panc1, Colo357 and Mia-PaCa2 pancreatic cancer cell lines, but not of the human non-cancer cells HEK-293 and treatment of Panc1 cells with soluble klotho (5nM) reduced viability by 70%. Moreover, soluble klotho increased Panc1 sensitivity to the chemotherapeutic drug 5-fluorouracil (5-FU). While 5-FU reduced viability by 50%, the combination reduced viability by 90%. Overexpressed and soluble klotho inhibited activation of the IGF-I pathway in pancreatic cancer cells, resulting in reduced phosphorylation of the IGF-IR and its downstream targets. Klotho also inhibited bFGF stimulation in pancreatic cancer cells, as evidenced by decreased FRS2 α and ERK1/2 phosphorylation. The in vivo activity of klotho was tested using a xenograft model. Panc1 cells were injected into both flanks of athymic mice and treated with daily intraperitoneal injections of either a control vehicle, 10 or 25 μ g/kg soluble klotho (5 mice per group). Treatment reduced tumor size by 30% to 50% respectively. Klotho administration did not affect weight or general health of the mice.

Conclusions: In conclusion, we show high klotho expression in normal pancreas and reduced expression in pancreatic cancer, discover klotho as a growth inhibitor of pancreatic cancer cells and identify it as an inhibitor of the IGF-1 and bFGF pathways in these cells. Importantly, klotho was able to reduce tumor growth in vivo. These data suggest klotho as a novel tumor suppressor. As soluble klotho is an endogenous hormone, its use as a novel therapy may be feasible and should be further explored.

In vivo effect of baicalein on human adrenal cortex carcinoma xenograft nude mice model.

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Introduction: Adrenocortical carcinomas [ACC] are highly malignant tumors for which no effective medical treatment currently exists. The lack of a suitable animal model for these tumors has been a major obstacle in the evaluation of new therapeutic agents. We showed that 12LOX-p a lipoxygenase enzyme, which seems to play a key role in the pathogenesis of cancer, is expressed in H295R and in 3 primary human ACC cells. Moreover, baicalein a 12-LOX inhibitor was shown to induce apoptotic cell death in these cells. The aim of this study was to establish and characterize a subcutaneous (sc) xenograft model of human adrenal carcinoma, and to study the effect of baicalein treatment on tumor mass and treatment-related toxicities.

Methods: Eight female nude mice were sc injected with 12 million H295R cells. In 7 mice, tumors became visible 31-38 days after injection. Five animals received baicalein 5mg/day in oil and 3 served as controls and received oil by gavage.

Results: In 3 animals, tumor size was larger than 10mm (late stage), and in 2 it was 3mm (early stage) when baicalein was initiated. The tumor growth was very slow in the 2 animals who received baicalein at an early stage [3mm]. On the other hand, no remarkable effect was seen in the growth rate of tumors which were at a late stage when treatment was initiated. It took 2 weeks to reach maximal tumor size in the control and late stage baicalein treatment group in contrast to 6 weeks in those who received early treatment. Blood cortisol was 3.17 ± 1 mcg/dl in mice with tumors and below 1mcg/ml in the mice devoid of tumors. Pathologic examination showed aggressive ACC tumor with local invasion into blood vessels, and muscle and lung metastasis. There was no sign of baicalein toxic effect in the kidneys, liver, spleen, heart, pancreas and intestine.

Conclusions: In this preliminary study we describe a model of aggressive and functional human ACC. Baicalein treatment given by gavage at early stage is effective in slowing ACC tumor growth induced in nude mice. More studies are needed to confirm the results and to reveal whether baicalein has static or apoptosis-induction effect on tumor cells in vivo.