Lunasin: A Cancer-Preventive Soy Peptide
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Lunasin is a novel, cancer-preventive peptide whose efficacy against chemical carcinogens and oncogenes has been demonstrated in mammalian cells and in a skin cancer mouse model. Isolated and characterized in soy, lunasin peptide is also documented in barley. Lunasin is found in all of the genotypes analyzed from the US soy germ plasm collection and in commercially available soy proteins. Pilot studies show that lunasin is bioavailable in mice and rats when orally ingested, opening the way for dietary administration in cancer prevention studies. Lunasin internalizes into mammalian cells within minutes of exogenous application, and localizes in the nucleus after 18 hours. It inhibits acetylation of core histones in mammalian cells. In spite of its cancer-preventive properties, lunasin does not affect the growth rate of normal and established cancer cell lines. An epigenetic mechanism of action is proposed whereby lunasin selectively kills cells being transformed or newly transformed by binding to deacetylated core histones exposed by the transformation event, disrupting the dynamics of histone acetylation-deacetylation and leading to cell death.

Key words: lunasin, soy, soybean, cancer, peptide

Introduction
The consumption of soybean products is associated with overall low mortality rates from cancers of the prostate, breast, colon, and others.1,2 Southeast Asians have a 4- to 10-fold lower incidence of and death from breast and prostate cancers. However, following emigration to the United States, the risk of these cancers rises rapidly in one generation to that equal to that of Americans.2,3 Differences in the diet are thought to account for a large part of this variation.4 The average intake of soy protein in Asia varies from 10 g/d in China to 30 to 50 g/d in Japan and Taiwan.5 In contrast, Americans eat less than 1 to 3 g/d. Two-thirds of the reported studies in the epidemiological literature associate soy intake with reduction of cancer risk.6 More recent epidemiological studies, animal experiments, and in vitro studies also show that soy products reduce cancer risk.7

A study of prostate cancer mortality in 42 countries showed that grains and cereals are protective against prostate cancer, and that soy products seem to be a major protector.8 Soy products are associated with decreased risk for prostate,9,10 breast,1 and endometrial cancers.12 Consumption of soy milk more than once a day is associated with a 70% reduction in prostate cancer risk compared with no soy milk intake.10 Candidate chemopreventive substances in soy include the Bowman-Birk protease inhibitor (BBI) and a BBI-enriched soy concentrate (BBIC), inositol hexaphosphate (phytic acid), β-sitosterol, and isoflavones.13-14 BBIC and isoflavones are the most widely studied.15-16 BBIC, now in human clinical trials for oral cancer prevention, has been shown to be protective against a number of cancers in in vitro and in vivo models, including models of prostate cancer.15-19 BBIC evidently works by inhibiting proteases involved in initiation and promotion of carcinogenesis,19 but the molecular details remain to be established.

The administration of soy isoflavones in a soy protein matrix has raised the possibility of a contribution of other proteins to the observed preventive effects attributed to isoflavones. Lobund-Wistar rats fed a diet containing soy meal (50% protein) had a 30% incidence of prostate tumors compared with a 3% incidence in rats fed soy protein isolate (>90% protein), although both of these substances have approximately equal isoflavone content.20 Soy protein isolates with negligible isoflavones are more effective than isoflavone-enriched soy in reducing mammary tumors in rats,21-22 suggesting that the mechanism of the preventive effect of soy isolate is
distinct from that of the isoflavones. These observations give special significance to the discovery of the cancer-preventive properties of lunasin and its presence in soy proteins.

**Discovery of the Anti-Mitotic Effect of the Lunasin Gene**

In a project to enhance the nutritional quality of soy protein through bioengineering, a gene coding for a soy albumin protein (GM2S-1) was cloned. GM2S-1 codes not only for the methionine-rich protein that was sought but for three other proteins; a signal peptide, lunasin, and a linker peptide. Lunasin is a 43-amino acid peptide that contains a poly-D carboxyl end with 9 D-residues (underlined), an −RGD- cell adhesion motif (bold), and a predicted, structurally conserved helix region (underlined italics):

SKWQHQQDSCRKQLQG.vnLTPCEKHIMEKIQGRGDDBDDDDDD.

Transformation of the lunasin cDNA into *Escherichia coli* leads to arrest of cell division manifested in non-septated filaments. The cells transformed with full lunasin fail to divide, while those transformed with the deletion mutant control without the poly-D divide normally. Subsequently, constitutive expression of lunasin tagged with green fluorescent protein in mammalian cells (murine hepatoma, human breast cancer cells, and murine fibroblast) leads to mitotic arrest and lysis of cells containing broken pieces of chromosomes to which the lunasin is attached. The lunasin evidently binds to the kinetochore of the centromere, a hypoacetylated region of the chromosome, and prevents attachment of the microtubule to the centromere, resulting in mitotic arrest. This is the first indication that lunasin, a highly negatively charged molecule, could bind specifically to the positively charged deacetylated histones in the hypoacetylated regions of the chromatin. Thus, the constitutive expression of the lunasin gene in mammalian cells leads to cell death, suggesting that it has a possible use in cancer therapy. However, since the lunasin gene affects both normal and cancer cells, a specific delivery system that targets cancer cells is needed.

**The Cancer-Preventive Property of the Lunasin Peptide**

Since people eat proteins and peptides in soy, the cancer-preventive property of lunasin peptide was next investigated. In contrast to the anti-mitotic effect of the constitutive expression of the lunasin gene, lunasin peptide added exogenously prevents transformation of mammalian cells caused by chemical carcinogens (DMBA and MCA) and viral oncogenes (E1A and ras). Using the foci formation assay, lunasin suppresses transformation by about 62% to 90% relative to the positive control (DMBA or MCA alone) at concentrations ranging from 10 nM to 10 μM. On a molar basis, lunasin is more effective than BBIC, a known cancer-preventive agent from soy. Lunasin also suppresses transformation of NIH3T3 cells induced by E1A in a dose-dependent manner, with an effective dose as low as 20 nM. Interestingly, lunasin is effective even when added up to 15 days after transfection with E1A gene, suggesting its efficacy when applied even after the transformation event. How this translates into animal models in terms of lunasin administration relative to the time after application of the carcinogen remains to be shown.

In the first animal model, lunasin applied topically at 250 μg/week suppresses skin papilloma formation in SENCAR mice treated with DMBA and TPA by 70% compared with the untreated control. Tumor multiplicity (tumors/mouse) is also reduced, and the appearance of papilloma is delayed by 2 weeks in the mice treated with lunasin relative to the untreated control. This is consistent with a recent observation that lunasin slows down epidermal cell proliferation in mouse skin in the absence and presence of DMBA using a 3H2O-labeling method to measure cell proliferation in vivo.

**Molecular Mechanism of Action**

Interestingly, lunasin peptide added exogenously to mammalian cells internalizes within a few minutes and localizes in the nucleus in approximately 18 hours. There is also evidence that lunasin is found in hypoacetylated regions of the chromosome such as the telomere. For internalization, the −RGD-cell adhesion motif is required in one cell line (C3H), but is unnecessary in another line (NIH3T3), suggesting that the role of −RGD- is cell-line specific. How lunasin internalizes into the cell and ends up in the nucleus is an intriguing mechanism that remains to be elucidated.

Lunasin inhibits core (H3 and H4) histone acetylation in mammalian cells, both normal (C3H) and cancerous (MCF-7), in the presence of the deacetylase inhibitor sodium butyrate (Figure 1). This is supported by in vitro binding studies showing that lunasin binds specifically to deacetylated core histones but not to acetylated histones. The poly-D is required for binding, while the putative helical region enhances binding but is not required. This helical region is structurally conserved and has homology with a number of chromosome-binding proteins, suggesting that it might target lunasin to core histones.

A common property of cancer-preventive agents is their ability to slow down growth of cancer cells but not normal cells. In contrast, lunasin does not affect the growth of either normal or established cancer cell
Interestingly, BBIC does not affect growth of normal cells either. Lunasin is still effective when added 15 days after transfection with E1A, suggesting that it is effective only within a certain window of the transformation event. A working mechanism based on the E1A-Rb-HDAC model is proposed (Figure 2).

The mechanism shows that lunasin selectively kills cells that are being transformed or newly transformed by binding to deacetylated histone substrates exposed by the transformation event and by inhibiting histone acetylation catalyzed by histone acetyl transferases. This disrupts the dynamics of histone acetylation-deacetylation, which is perceived as abnormal by the cell and leads to cell death. In normal and established cancer cell lines, this window is absent because the deacetylated histones are not accessible functionally and/or physically. An analogy is that lunasin is a “watchdog” agent that sits in the nucleus and effectively does nothing when there is no transformation event. When a transformation event occurs, lunasin is triggered into action and binds to the deacetylated core histones exposed by the transformation event, leading to the selective killing of cells being transformed or newly transformed. It is likely that this model will evolve as the mechanism is better understood.

Chromatin and cancer were linked when the tumor suppressors Rb and p53 were shown to recruit histone deacetylase (HDAC) to maintain core histones that interact with transcription factors in the hypoacetylated state to repress genes involved in carcinogenesis. Specific viral oncoprotein sequences disrupt the interaction between Rb and HDAC1 and displace histone deacetylase activity from Rb. This is significant because viral oncogenes target and inactivate critical components of tumor suppressor proteins such as Rb and p53, which are mutated in 20% to 50% of advanced-stage prostate cancers.

To summarize, the two major tumor suppressor proteins, Rb and p53, utilize HDACs as co-repressors of transcription. This repression is lost when viral oncogenes such as E1A and HPV disrupt the interaction between HDACs and Rb and p53, leading to proliferation of transformed cells. Mutations in Rb and p53 have been associated with a number of human cancers. It is conceivable that lunasin, when introduced into the tissues, could act as a surrogate tumor suppressor by inhibiting histone acetylation. The fundamental nature of this epigenetic mechanism suggests that lunasin could be effective against different kinds of cancer where chromatin modification is involved in carcinogenesis. Clearly, demonstrating the efficacy of orally administered forms of lunasin against different cancers is a priority.

Lunasin in Soy and Other Seeds

We propose an intriguing role of lunasin in seed development. The three stages of angiosperm seed development are rapid cell division and differentiation, followed by cessation of cell division in the central parenchyma cells of the cotyledon or endosperm and enlargement of the cells accompanied by biosynthesis of storage forms of carbohydrates, proteins, lipids, and nucleic acids for the germinating seeds. In the last stage, the seed dehydrates. The second stage is considered unique to angiosperm seeds in which there is “endoreduplication” of DNA without cell division. Endoreduplication is a unique cell cycle wherein the G1- and S-phases occur without cell division, and thus DNA synthesis is uncoupled from cell division, allowing DNA accumulation for purposes of storage. We propose that lunasin is an effector molecule that allows arrest of cell division and initiates the second stage of seed development. Therefore, in theory, all angiosperm seeds should contain lunasin. Lunasin has been found and characterized in barley and has also been found in wheat (unpublished data). An initial screening using Western blot shows that it is not detected in the common bean. A more rigorous and systematic screening should be carried out, which could include different extraction procedures, testing different stages of seeds, and perhaps designing antibodies to recognize lunasin homologs.

Lunasin extracted from soy has been characterized. A recent screening of the US soybean germ plasm collection at the University of Illinois shows that lunasin is present in all genotypes in varying...
amounts, suggesting the possibility of selecting and breeding varieties of soy with higher lunasin content. The presence of lunasin in all commercially available soy protein samples analyzed suggests the presence of lunasin in most commercial soy products. Interestingly, lunasin is a very heat-stable peptide, Figure 2. E1A-Rb-HDAC model to explain the ability of lunasin to suppress E1A-induced transformation without affecting growth of immortalized and established cancer cell lines. Rb controls G1/S transition by interacting with E2F promoter and recruiting HDAC to keep the core histones in the deacetylated (repressed) state. Top diagram: Cells being transformed or newly transformed. In the presence of lunasin, E1A inactivates Rb and dissociates the Rb-HDAC complex, exposing the deacetylated core histones in the E2F promoter. Lunasin competes with histone acetyl transferases (HAT) in binding to the deacetylated core histones. Lunasin binds and turns off transcription, perceived as abnormal by the cell and commits apoptosis. HAT binds and acetylates core histones, turning on E2F cell cycle transcription factors. Middle diagram: In immortalized, non-tumorgenic cells, the active Rb-HDAC complex keeps the core histones in E2F promoter deacetylated and inaccessible to added lunasin. Bottom diagram: In established cancer cell lines, transformation has occurred in the absence of lunasin. HAT has acetylated the core histones, turning on cell cycle transcription factors and keeping the acetylated core histones inaccessible and unable to react with added lunasin.
Oral Bioavailability of Lunasin

One of the properties of an ideal cancer-preventive agent is that it can be taken orally. A crucial question is whether lunasin, a peptide, survives digestion in the gastrointestinal tract when ingested orally. Pilot studies using $^3$H-labeled synthetic lunasin showed that about 35% of the oral dose is absorbed and ends up in the various tissues of mice and rats 6 hours after administration by gavage (unpublished data). Furthermore, lunasin from the blood and liver is intact and is bioactive by an in vitro assay. Bioavailability studies are continuing.

Future Work

The epigenetic mechanism of lunasin suggests that it could be effective against many different cancers where chromatin modification is involved in carcinogenesis. The preventive efficacy of lunasin administered in the diet and other routes should be tested against breast, prostate, colon, lung, oral, and cervical cancers. The proposed epigenetic mechanism should be further elucidated through genomics and proteomics. The intriguing mechanism of how lunasin enters the cells and localizes in the nucleus should also be investigated. Further oral bioavailability studies are being carried out.

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