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Conference on 'Nutrition and health: cell to community'

Symposium 1: Nutrition and epigenetics
Epigenetic modifications and human pathologies: cancer and CVD

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Epigenetic changes are inherited alterations in DNA that affect gene expression and function without altering the DNA sequence. DNA methylation is one epigenetic process implicated in human disease that is influenced by diet. DNA methylation involves addition of a 1-C moiety to cytosine groups in DNA. Methylated genes are not transcribed or are transcribed at a reduced rate. Global under-methylation (hypomethylation) and site-specific over-methylation (hypermethylation) are common features of human tumours. DNA hypomethylation, leading to increased expression of specific proto-oncogenes (e.g. genes involved in proliferation or metastasis) can increase the risk of cancer as can hypermethylation and reduced expression of tumour suppressor (TS) genes (e.g. DNA repair genes). DNA methyltransferases (DNMT), together with the methyl donor S-adenosylmethionine (SAM), facilitate DNA methylation. Abnormal DNA methylation is implicated not only in the development of human cancer but also in CVD. Polyphenols, a group of phytochemicals consumed in significant amounts in the human diet, effect risk of cancer. Flavonoids from tea, soft fruits and soya are potent inhibitors of DNMT *in vitro*, capable of reversing hypermethylation and reactivating TS genes. Folates, a group of water-soluble B vitamins found in high concentration in green leafy vegetables, regulate DNA methylation through their ability to generate SAM. People who habitually consume the lowest level of folate or with the lowest blood folate concentrations have a significantly increased risk of developing several cancers and CVD. This review describes how flavonoids and folates in the human diet alter DNA methylation and may modify the risk of human colon cancer and CVD.

Epigenetics: Human disease: Colon Cancer: Atherosclerosis: Nutrition: Folate: Polyphenols

Epigenetics and regulation of gene activity

Epigenetics is defined as processes that act to regulate heritable changes in gene activity that are transmitted through meiosis and mitosis but that are not accompanied by changes in the DNA coding sequence. Epigenetic signals (or marks) control gene expression through re-modelling of chromatin. Chromatin consists of DNA and DNA-binding proteins including histones, which interact to form repetitive nucleoprotein units called nucleosomes. Epigenetic control of gene expression is critically important in regulating embryonic development, cellular differentiation and organogenesis and for key biological processes such as imprinting, and silencing of large chromosomal domains such as the X-chromosome in females[1]. Conversely, dysregulation of epigenetic processes may be causal for several human diseases including cancer and heart disease[1,2] (Fig. 1).

Epigenetic mechanisms include nucleosome remodelling, histone modifications (including acetylation, methylation and ubiquitination), regulatory non-coding (small and micro) RNA and DNA methylation. These are linked

Abbreviations: EGCG, (−)-epigallocatechin 3-gallate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; DNMT, DNA methyltransferase; MMP, matrix metalloproteinase; SMC, smooth muscle cell; TS, tumour suppressor.

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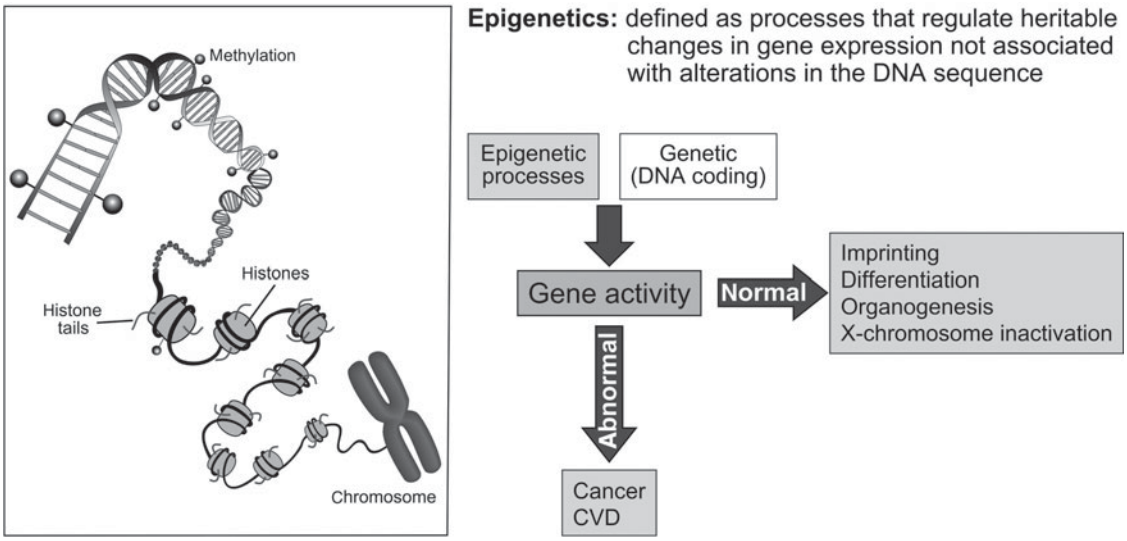

Fig. 1. Epigenetics, diet and human disease. A simplified scheme illustrating the structure of mammalian chromatin and defining the role of epigenetic modifications both in normal human development and disease.

processes and all contribute to controlling gene activity by changing chromatin architecture and/or access by transcription factors[1].

Dysregulation of any of these processes has severe consequences for the phenotype and function of the cell. During malignant transformation, cancer cells can exhibit global changes in the chromatin structure affecting the whole epigenome, altering the expression of several hundreds of genes and perturbing entire metabolic pathways. Consequently, it has been proposed that epigenetic dysregulation can be as detrimental to the cell as mutations in DNA coding[1].

Epigenetics, diet and disease

Although epigenetic marks remain comparatively stable during mitotic transmission, they are influenced by ageing and by the environment. In an elegant study of monozygotic twins aged between 3 and 74 years (*n* 40 pairs), genome-wide cytosine methylation, gene-specific DNA methylation and histone acetylation (H3 and H4) patterns in lymphocytes were found to correlate strongly in young twins, but these associations weakened both with age and depending on how divergent the twin's health and lifestyles had become[3]. Approximately one-third of twins harboured epigenetic differences. Global gene expression, measured by microarray analysis, differed more that fourfold in the oldest twin pair compared to gene activity in the youngest[3].

Diet can profoundly alter epigenetic patterns in animals. The Agouti mouse has been used extensively to investigate the impact of maternal nutrition on the fetal epigenome and the phenotype of the offspring. In this model, coat colour is linked to the methylation status of the agouti gene, which is highly dependent on maternal diet. Most importantly, altered epigenetic marking is associated with an increased risk of diseases including a diabetes-like condition, obesity and tumorigenesis[4,5]. The influence of diet on epigenetics and fetal programming in relation to health and disease will be covered in greater detail in a subsequent paper (KA Lillycrop, in this issue).

How diet contributes to epigenetic control of gene expression and human disease risk is poorly understood. It is acknowledged that abnormal epigenetic control of gene expression increases disease risk (certainly for cancer) and that diet can also profoundly influence risk. What remains to be established is a causal link between diet and epigenetics in the development of human disease and whether diet is differentially effective at various stages in the human lifespan.

Certain bioactive food components, such as sulforaphanes from broccoli, diallyl disuphides from garlic and resveratrol in wine, have been shown (*in vitro* and *in vivo*) to alter epigenetic processes with positive consequences for cell function, including control of proliferation, upregulated apoptosis and a reduction in inflammation[6]. All of these food components are associated with a reduced risk of human cancer at various sites. Inhibition of histone deacetylase activity increases the transcription factor access to DNA and induces gene reactivation. Isothiocyanates present in cruciferous vegetables inhibit histone deacetylase activity in colon, breast and prostate cancer cells *in vitro*. Sulforaphane, a powerful inducer of phase 2 detoxification enzymes, increases histone acetylation and inhibits intestinal polyp formation in Apc min mice. Moreover, histone deacetylase activity (H3 and H4) is rapidly inhibited (3–6 h) in blood cells from human volunteers fed a relatively small single bolus (68 g) of broccoli sprouts (reviewed in[7]). Conversely, diallyl disulfide from garlic increases H3 and H4 histone acetylation, tumour suppressor (p21) gene expression and inhibits colon cancer cell growth in culture (reviewed in[8]). The polyphenols curcumin (from the spice turmeric) and resveratrol (from red wine), which have strong antioxidant, anti-inflammatory and anti-carcinogenic properties, also modify histone acetylation patterns in circulating inflammatory cells and induce histone deacetylase activity[9].

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In contrast, the impact of diet on recently identified epigenetic processes, for instance, endogenous non-coding or regulatory (small and micro) RNA that suppress gene activity at the transcriptional level and where dysregulation may increase the risk of cancer remain unexplored[10].

Most studies investigating how diet alters the epigenome have explored how dietary components alter DNA methylation.

Aberrant DNA methylation and abnormal gene expression

Post-replicative DNA methylation in mammals involves addition of a 1-C methyl group at the fifth carbon position of cytosine residues within CpG dinucleotides. S-adenosylmethionine (SAM) acts as methyl donor in this reaction, which is catalysed by DNA methyltransferase (DNMT) enzymes. DNMT 3 (3a and 3b) regulates *de novo* methylation during development, whereas DNMT 1 maintains DNA methylation patterns during cell replication. Approximately 4% of cytosines in DNA are modified to 5-methylcytosine, with most occurring in the sequence, CpG. The genome methylation pattern is precisely inherited during mitosis and is highly tissue specific and species specific. Cytosine methylation changes the structure of the major groove in the DNA molecule and disrupts the attachment of DNA-binding proteins and transcription factors. In general, genes methylated at specific sites (for example upstream of a promoter region) are either not transcribed into mRNA or are transcribed at a reduced rate, and translation into the protein for which the gene encodes is reduced. Epigenetic DNA methylation therefore contributes to the control of gene and ultimately protein expression[11].

Most information on how dysregulated DNA methylation affects human disease risk comes from research into cancer. Two types of aberrant DNA methylation are universally present in tumours. DNA, both from benign and malignant carcinomas, is substantially undermethylated (hypomethylated) compared to DNA from adjacent normal tissue. DNA hypomethylation is associated with increased transcription and expression of proto-oncogenes that stimulate malignant cell growth. Genome-wide DNA hypomethylation occurs early in carcinogenesis and precedes mutation and deletion events. Conversely, increased DNA methylation (hypermethylation) is associated with gene silencing. Hypermethylation of cytosines in the promoter region of tumour suppressor (TS) genes (for example DNA repair genes and genes that down-regulate cell proliferation) is linked with cancer progression. Gene-specific DNA hypermethylation occurs both in early and advanced stages of malignant transformation. Aberrant DNA methylation and gene expression patterns may be causal in tumorigenesis[12].

Many bioactive food compounds including folate, selenium, flavonoids, alcohol and fatty acids are associated with an altered risk both of human colon cancer and heart disease. All of these dietary components modify cytosine methylation. The impact of several of these phytochemicals and micronutrients on DNA methylation in colon carcinogenesis are reviewed comprehensively elsewhere[13,14].

The remainder of this review describes how specific dietary polyphenols reactivate TS genes by reversing DNA hypermethylation and the consequence of this for risk of cancer and how the vitamin B folate modifies cytosine methylation in relation to both heart disease and colon carcinogenesis.

Polyphenols, DNA methyltransferase activity and tumour suppressor gene re-expression

Hypermethylation of CpG islands in the gene promoter contributes to gene silencing. TS gene hypermethylation is a universal event in early cancer, with more genes becoming hypermethylated as the disease progresses. Drugs such as 5-aza-2′-deoxycytidine (decitabine) that reverse DNA hypermethylation and re-establish TS gene expression are used in cancer therapy. However, they are genotoxic to normal cells. Maintenance of normal DNA methylation and gene expression patterns by dietary phytochemicals and reversal of methylation-induced inactivation of TS genes may be an alternative approach for the prevention and treatment of cancer.

Polyphenols, a group of plant phytochemicals consumed in significant amounts in the human diet modulate genomic stability and risk of cancer *in vitro* and *in vivo*[15–17]. There is strong evidence that polyphenols from tea, soft fruits and berries, vegetables, apples, and even from wine, are potent anti-carcinogenic agents *in vitro* and in animal models that prevent DNA instability at several sites in the carcinogenic pathway[15–17]. Genoprotective mechanisms include modulation of carcinogen metabolism (either by inhibiting the activation of cytochromes P450 or by inducing detoxification by glutathione transferase and glucuronyltransferase), decreased binding of the carcinogen to DNA, inhibition of oxidative DNA damage, alteration in cell signalling and gene expression, reduced inflammation, increased apoptosis and inhibition of malignant transformation, cell invasiveness, angiogenesis and metastasis[15]. Moreover, several polyphenols are potent inhibitors of DNMT activity *in vitro*, capable of reversing DNA hypermethylation and reactivating TS gene activity. Polyphenols inhibit DNMT activity and DNA methylation in two ways. First, by direct insertion into the binding pocket of DNMT (competitive inhibition) and second, indirectly by decreasing intracellular SAM concentrations and non-competitively inhibiting DNMT activity[18]. Tea and soya are two of the most widely consumed plant products worldwide. Tea components (especially green and black tea) and specific soya isoflavones inhibit DNMT activity in human cancer cells. Genistein from soyabean and (−)-epigallocatechin 3-gallate (EGCG) from green tea are the most potent DNMT inhibitors. EGCG (and other green tea metabolites) dose-dependently inhibit DNMT activity in human oesophageal cancer cells[18]. Molecular modelling reveals that the gallate moiety on the D ring of EGCG interacts strongly with the cytosine-active site on the DNMT enzyme. Additionally, hydrogen bonds formed between hydroxyl groups of the A and B rings and Sr1229

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and Cys 1225 on the protein contribute to the high-affinity binding of EGCG and inhibition of activity[18]. Genistein (from soya) was also found to interact strongly with DNMT, dose-dependently inhibiting enzyme activity[18]. Certain polyphenols are substrates for catechol O-methyltransferase, which is structurally analogous to DNMT. SAM is consumed in the reaction and intracellular S-adenosylhomocysteine (SAH), a potent inhibitor of DNMT is elevated. Polyphenols including EGCG, quercetin, myricetin and fisetin may decrease DNA methylation by altering the cellular ratio SAM:SAH and indirectly inhibiting DNMT activity. There is currently little evidence in support of this mechanism. Feeding mice green tea extract containing EGCG (65% (v/v) for 7 d) or transgenic Apc min mice (0·16% (v/v) EGCG for 9 weeks) did not alter intestinal or liver SAH[18].

DNMT 1 inhibition demethylates CpG islands in the promoter regions of silenced TS genes including $p16^{ink4a}$, retinoic acid receptor β, methylguanine methyltransferase, mMLH1 and glutathione S-transferase π in cancer cells. Reactivation of TS genes is associated with a corresponding increase in mRNA and protein expression. EGCG (20–50 μM for up to 6 d) reverses DNA hypermethylation and increases expression of methylguanine methyltransferase, p16 and hMLH1 in cultured KYSE 510 oesophageal cancer cells. Changes in cytosine methylation and mRNA expression were detected after only 48 h exposure to the green tea polyphenol and were progressive with time. Moreover, reversal of DNA hypermethylation was associated with increased protein expression (determined by Western blotting). EGCG can also reactivate retinoic acid receptor β in prostate and breast cancer cells, p16 in colon cancer cells and glutathione S-transferase π in prostate cells[19–21]. Genistein (at the same concentrations) partially reversed DNA hypermethylation and reactivated p16, retinoic acid receptor β and methylguanine methyltransferase gene expression[22]. The isoflavones biochanin A and daidzein and the flavonoids, myricetin, quercetin, hesperitin, naringenein, apigenin and luteolin were active, but less effective at altering DNA methylation and reactivating TS gene expression[22]. Reactivation of TS gene expression by polyphenols is associated with reduced cancer cell progression. Malignant transformation involves phenotypic changes in initiated cells that promote invasion into extracellular tissue matrices. The TS matrix metalloproteinase (MMP) inhibitor RECK inhibits angiogenesis, invasion and metastasis. RECK activity is down-regulated in several human cancers and is associated with promoter hypermethylation in the gene. In an elegant study, Kato *et al.*[23] investigated how EGCG altered RECK DNA methylation and expression in four human oral squamous cell carcinoma cell lines. Critically, they investigated whether reversing hypermethylation and increasing RECK gene expression could inhibit oral squamous cell carcinoma migration and invasiveness. EGCG (20–50 μM for up to 6 d) dose- and time-dependently decreased RECK DNA methylation and increased mRNA in two of the four cell lines. Significantly, this was associated with a subsequent decrease in MMP-2 and MMP-9 expression and in the ability of the cancer cells to invade a three-dimensional collagen model. Cell proliferation, depth of migration and frequency and size of invasive foci were all reduced by EGCG[23]. However, the ability of EGCG to decrease DNA hypermethylation and reactivate TS gene expression and activity is inconsistent, with some studies reporting no effect of EGCG on DNA methylation in several human cancer cell lines[24,25].

Evidence for a modulating effect of dietary polyphenols on DNA methylation and re-expression of TS genes *in vivo* is less convincing. Consumption of genistein by mice appears to correlate positively with changes in prostate DNA methylation at CpG islands[26]. Conversely, feeding a composite of green tea polyphenols (0·1, 0·3 and 0·6% (v/v) in drinking water for 12 or 24 weeks) to wild-type or TRAMP (transgenic adenocarcinoma of mouse prostate) mice did not change normal or cancer-specific DNA methylation patterns either in target prostate tissue or in gut and liver tissue[27]. In this model, genome-wide DNA hypomethylation occurs at an early stage in cancer progression with loci-specific hypermethylation detected in late-stage disease. No significant dose- or time-dependent effect of GTP on either global- or gene-specific DNA methylation (measured by several different biomarkers) was observed[27].

In general, the concentrations of polyphenols employed to inhibit DNMT activity both *in vitro* and *in vivo* are considerably higher than can be achieved nutritionally. The levels of EGCG shown to be effective in reversing DNA hypermethylation and TS gene silencing (generally 10–50 μM) are 50-fold higher than blood and tissue concentrations measured after tea drinking[18]. The highest dose of GTP studied in the TRAMP mouse model (0·6% (v/v)) is equivalent to a human drinking eighteen cups of green tea per day. Similarly, the concentration of genistein needed to alter cytosine methylation and TS gene activity (5–20 μM) are 3–10-fold higher than can be achieved by eating soya products[18]. Dietary polyphenols are rapidly metabolized intracellularly through several biochemical pathways, including glucuronidation, sulfation and methylation. The lack of effect of polyphenols on DNMT activity, DNA methylation and gene expression *in vivo* is probably due to limited functional bioavailability post-metabolism. Whether dietary polyphenols can modify DNA methylation and TS gene expression in human subjects is unknown, but it is doubtful whether these compounds, consumed at nutritional levels, will have a major impact on DNA methylation and gene reactivation.

Folate deficiency and human diseases

Folates, a family of water-soluble B vitamins, play a crucial role in the development of human diseases such as cancer and heart disease in adults, cognitive dysfunction and dementia in the elderly and congenital defects in babies[28–30]. Suboptimal folate status is widespread with 40% of 15–18 year olds in the UK exhibiting marginal folate status and overt folate deficiency common in people over 65 years of age, especially in the institutionalized elderly.

Low-folate status has been implicated in cancer development, notably of the cervix, lung, breast, brain,

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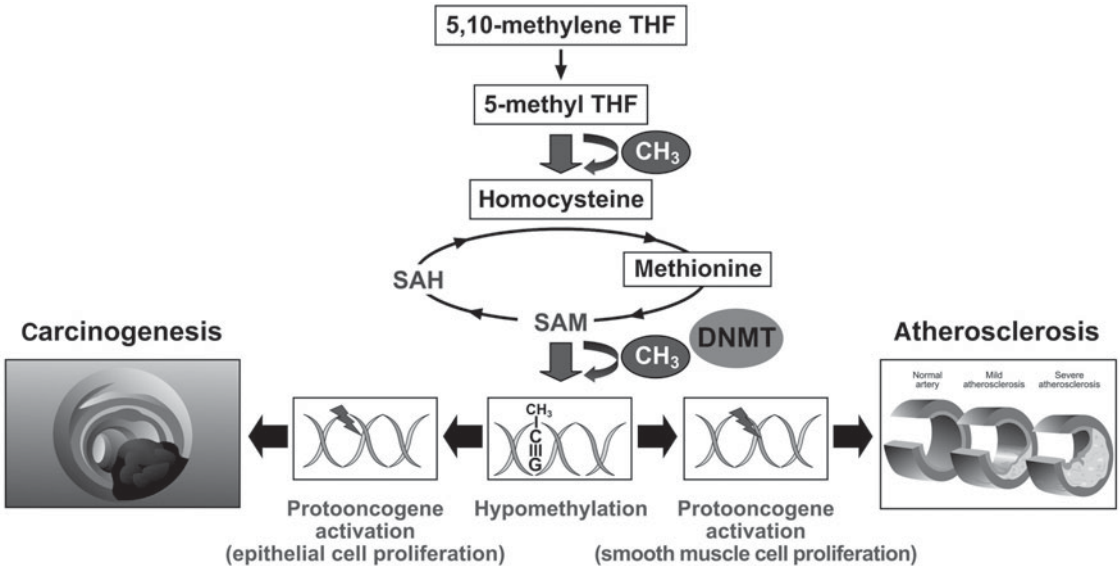

Fig. 2. Folate deficiency, cancer and CVD: a common mechanism? A simplified scheme describing how folate deficiency may alter normal DNA methylation and how this could impact both on risk of colon cancer and atherosclerosis.

colorectum and pancreas. The evidence linking folate deficiency and human cancer is strongest for the colon. Data from the majority of human studies (retrospective, case–control and prospective) suggest that individuals with the highest habitual folate intake or with the highest circulating folate concentrations have a reduced risk of developing colon polyps or tumours[31–33]. Folate acts to maintain genomic stability by regulating DNA biosynthesis, DNA repair and DNA methylation. Folate deficiency both initiates and accelerates cancer by disrupting each of these processes.

Poor folate status is also associated with an increased risk of CVD. Historically, this has been attributed to the metabolic relationship between low folate and high homocysteine (hyperhomocysteinemia). Adverse effects of homocysteine include impairment of endothelial and smooth muscle cell (SMC) function, changes to vasodilation, altered extracellular matrix biochemistry, endoplasmic reticulum stress and increased coagulation and impaired fibrinolysis[34]. Most human observational studies support a positive relationship between homocysteine and CVD[35–37]. However, several recent large intervention trials have failed to find any benefit of homocysteine-lowering therapies against death from cardiovascular causes in patients with pre-existing vascular disease or diabetics[38]. Consequently, there is considerable debate whether homocysteine is causal for vascular dysfunction[36]. In two recent prospective studies of men without prior CVD, high-serum folate was independently and significantly associated with a reduced risk of vascular events after adjusting for relevant confounders[39,40]. Blood homocysteine levels did not correlate with risk[40]. Several alternative theories of how low folate may independently increase CVD have been proposed. Folate can directly influence vascular endothelial cell function. Nitric oxide production and vasodilation is impaired in CVD. Folates are critical in maintaining nitric oxide production and preventing oxygen radical formation by physically stabilizing tetrahydrobiopterin in the conversion of L-arginine to L-citrulline and preventing endothelial nitric oxide synthase uncoupling. Folic acid supplementation improves endothelial cell function in hyperhomocysteinemic patients with coronary artery disease, in diabetics, in subjects with hypercholesterolemia and in healthy subjects fed a high methionine load[41,42]. However, in addition to deregulating vascular endothelial cells, low folate may perturb vascular SMC gene expression, growth and function by disrupting DNA methylation[2].

Folate deficiency, cancer and CVD: a common mechanism

Within the methionine cycle, 5-methyltetrahydrofolate remethylates homocysteine to methionine, which is subsequently metabolized to SAM. As described earlier, SAM serves as 1-C donor in the methylation of DNA. Under conditions of low-dietary folate, SAM concentrations are depleted causing hypomethylation of newly synthesised DNA and increased proto-oncogene expression (see aberrant DNA methylation and abnormal gene expression above). While abnormal DNA methylation and gene expression is a consistent event in tumorigenesis, aberrant cytosine methylation and proto-oncogene activation may also be a common mechanism linking malignant transformation in cancer and vascular dysfunction in heart disease (Fig. 2). The atherosclerotic plaque and the tumour are both monoclonal in origin with unregulated cell proliferation providing a growth advantage for selected clones. During atherogenesis, SMC (which make up approximately 90% of the aorta cell population) transform from quiescent, contractile cells to synthetic cells that migrate to the intima and rapidly multiply. Overexpression of growth factors and pro-proliferative oncogenes such as c-myc and

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p53 (which are commonly dysregulated in cancer) also drive SMC invasiveness and plaque formation. Moreover, dysregulated expression of genes involved in lipid accumulation, connective tissue formation, calcification and inflammation further accelerate plaque progression. Hence, changes in the phenotype and invasiveness of SMC in the vascular plaque are similar to changes in monoclonality and cell proliferation described for tumour formation[2].

Low-folate status is associated both with alterations in cytosine methylation in experimental and human studies (see later) and an increased risk of malignant transformation. Whether folate status can similarly influence DNA methylation and risk of vascular disease remains to be established.

The remainder of this review will describe evidence that abnormal DNA methylation may be causal in the development of atherosclerosis and that folate status influences genome-wide DNA methylation and the incidence of both colon cancer and vascular disease. How gene-specific DNA hypermethylation influences disease risk was covered in a paper also presented at this symposium (E Lund, unpublished results).

Folate, abnormal genome-wide DNA methylation and colon cancer

While extreme methyl donor depletion alters genome-wide cytosine methylation (primarily in the liver) and induces hepatocarcinogenesis in animal models[43,44], the effect of folate deficiency alone on global DNA methylation *in vitro* and *in vivo* is strongly influenced by the treatment regime, rodent species, tissue and genes examined (reviewed in[33]). Folate depletion decreases global DNA methylation in some human and animal cell lines *in vitro*, but not in others. Mouse NIH/3T3 fibroblast and CHO-K1 DNA became hypomethylated after 12 d in folate-depleted medium, but not in human colon adenocarcinoma HCT116 and Caco-2 cells[45]. Conversely, global and p53 TS gene DNA was hypomethylated in human colon adenoma cells grown in folate-depleted medium but was restored by folic acid repletion[46]. We have shown that global cytosine methylation is decreased in SV40-immortalised human colonocytes grown in folate-free medium for 14 d[47] but not in NCM460 non-malignantly transformed human colon cells under the same conditions[48]. Severe and prolonged folate depletion in rodents induces global DNA hypomethylation in the liver and certain regions of the colon[49–51]. Moderate folate deficiency does not consistently induce global DNA hypomethylation in blood, liver and colon, despite a significant depletion in blood and tissue folate and liver SAM[52–54].

Lymphocyte DNA is hypomethylated in women made experimentally folate deficient over several weeks[55,56] and low-dietary folate intake (<200 μg/d) correlates with hypomethylation of long-interspersed nucleotide element repeats (LINE-1; used as an indicator of global DNA methylation) in human colon tumours[57]. Conversely, LINE-1 methylation was not associated either with intake or blood folate in colon biopsy tissue collected from the Aspirin/Folate Polyp Prevention study[58]. Little association between folate and genome-wide DNA methylation is apparent in healthy individuals with sufficient blood folate[33,59,60]. The effect of supplementation with synthetic folic acid on DNA methylation is highly inconsistent and dependent on initial folate status, the level and duration of intervention, the genes and tissue reported and the health status of the subject[54]. DNA hypomethylation is reversed in lymphocytes from folate-deficient volunteers repleted with synthetic folic acid[55,56] and in leucocytes from colorectal adenoma patients given folic acid (400 μg/d for 10 weeks[61]). However, DNA methylation remained unchanged in the colonic mucosa of these patients[61]. Pharmacological (rather than nutritional) concentrations of folic acid (5–10 mg/d for 3–6 months) increased DNA methylation in the colorectum[62,63], but intervention with a more moderate dose (1 mg folic acid/d for 3 years) did not[58]. Supplementing healthy volunteers (with initial circulating folate concentrations within the normal range) has little impact on blood cell DNA methylation despite significantly elevated whole blood, plasma and lymphocyte folate[59,64].

Folate, DNA methylation and vascular disease

While there is good evidence that abnormal DNA methylation is causal for human carcinogenesis, decreased genomic methylation is also observed in cultured vascular cells and diseased vascular tissue. 5-methylcytosine levels drop rapidly (about 65%) in healthy rabbit aorta SMC cultured from aortic explants. Moreover, these cells change from a contractile to an invasive phenotype[65]. Hypomethylated DNA is also detectable in atherosclerotic plaques. Global genomic DNA is under methylated in advanced atherosclerotic lesions isolated from ApoE knockout mice and denuded New Zealand White rabbit aorta[65]. Most importantly, changes in DNA methylation patterns in mononuclear cells and in the aorta of ApoE mice occur early (4 weeks of age) and prior to appearance of vascular lesions, supporting a pathogenic role for abnormal DNA methylation in atherosclerosis[66]. Cytosine methylation is reduced (9%) in advanced human atherosclerotic lesions compared to normal arteries or arteries with fatty streaks and at a level similar to that detected in human tumours[65]. Both global genomic DNA and gene-specific DNA is hypomethylated in blood cells from uraemia patients (with hyperhomocysteinemia) compared to healthy subjects[60]. Gene-specific hypomethylation has also been measured in human atherosclerotic advanced lesions and is associated with expression of the SMC proto-oncogene platelet-derived growth factor[65] which may increase SMC proliferation in the atherosclerotic lesion. In addition to increasing SMC proliferation and invasiveness, DNA hypomethylation may also accelerate atherogenicity by up-regulating genes involved in lipid deposition and inflammation and increasing plaque instability. Several CpG islands are hypomethylated in the 15-lipoxygenase gene promoter and mRNA expression is correspondingly increased in human plaques[65]. As in cancer, gene-specific DNA hypermethylation is also present in atherosclerosis. Methylation of the estrogen

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receptor-α gene (which restricts cell proliferation) is increased (about two-fold) in arteries from patients with severe atherosclerosis compared to healthy subjects[67]. In addition, estrogen receptor-α hypermethylation is associated with a switch from a contractile to synthetic phenotype in human SMC explants[68]. Aberrant expression of other genes implicated in atherosclerotic plaque progression include interferon-γ, MMP-2, MMP-7, tissue inhibitor of metalloproteinases (TIMP)-3, p53 and EC-SOD (extracellular superoxide dismutase)[69].

Cancer and atherosclerosis are diseases of old age. Moreover, normal ageing is associated with altered DNA methylation patterns, making it difficult to clearly establish the effects of diet alone on DNA methylation and disease risk. Similarly, determining whether low folate induces plaque development through abnormal DNA methylation independently of homocysteine is complicated by the intimate metabolic relationship between these two metabolites. While there is evidence that homocysteine is associated with altered DNA methylation and vascular disease, currently there is only very limited evidence that folate alone alters cytosine methylation in atherosclerosis.

In heterozygous and homozygous variant methylenetetrahydrofolate reductase mice, folate concentrations are low, homocysteine is elevated and brain and ovary tissue DNA is hypomethylated. Lipid deposition is increased in the aorta of aged methylenetetrahydrofolate reductase variant mice[70]. Similarly, in mice heterozygous for the cystathionine-β-synthase gene, DNA methylation patterns in the imprinted H19 gene are changed in response to hyperhomocysteinemia[71]. However, here, alterations in methylation profiles are highly tissue specific and do not correlate consistently with H19 gene expression[71]. Plasma homocysteine and DNA methylation in peripheral mononuclear cells are negatively associated in healthy human subjects[72], while supraphysiological folate supplementation (15 mg/d for 8 weeks) reverses genome-wide DNA hypomethylation and abnormal gene expression (H19, IGF2) in hyperhomocysteinemic patients[60].

In an attempt to establish the independent effects of folate and hyperhomocysteinemia on vascular function, Brown *et al.*[73] developed an *in vitro* model of prolonged folate deficiency in cultured human endothelial (EA.hy926) cells[73]. Several intracellular folate vitamers were significantly depleted (up to 99%) but homocysteine, SAM and SAH were unchanged relative to cells grown in adequate folic acid. MCP-1, a cytokine up-regulated early in atherogenesis, was increased providing limited evidence that folate deficiency (independently of homocysteine) is associated with an atherogenic phenotype. However, global DNA methylation was unaltered and gene-specific methylation was not measured[73].

The effect of hyperhomocysteinemia and/or vitamin B deficiency on aortic plaque formation has been investigated extensively in rodent models. Generally, hyperhomocysteinemia is associated with a 2-fold increase in aortic plaque area[74–76] but vitamin B deficiency is not[74,77]. Conversely, vitamin B supplementation (up to three times control levels in the diet) is reported to decrease endogenous and homocysteine-induced plaque formation in ApoE null mice[74,76]. However, the data are inconsistent and profoundly dependent on the dietary regimen[74,76]. DNA methylation was not measured in any of these studies. We have recently developed an ApoE mouse model of folate and combined folate, vitamins B_6 and B_{12} deficiency that demonstrates either mild (2-fold increase in homocysteine) or moderate hyperhomocysteinemia (7-fold increase in homocysteine). Folate (but not combined vitamin B deficiency) significantly increased atherosclerotic plaque formation (approximately 17%) in the aorta of mice fed a high fat Westernised diet for 16 weeks. Moreover plaque area was not associated with plasma homocysteine. However, global DNA methylation in the mouse heart, aorta and liver was similar across all groups indicating that disease progression is probably not related to altered cytosine methylation in response either to vitamin B or hyperhomocysteinemia (SJ Duthie, unpublished results).

Conclusion

Diet strongly influences the risk of developing cancer and heart disease. Epigenetic changes are present in malignantly transformed tumour cells and in atherosclerotic lesions. Abnormal DNA methylation in target (epithelial or smooth muscle) cells is common to both diseases. Moreover, diet can modulate epigenetic marking and gene expression. There is some evidence that specific nutrients in the human diet that are strongly associated with risk of vascular disease and cancer can modulate DNA methylation.

Certain polyphenols, notably from green tea and soya, inhibit DNMT activity in cancer cells *in vitro* and reactivate tumour suppressor genes silenced by DNA hypermethylation. The data are weaker in animal studies. Polyphenols undergo extensive metabolism *in vivo* and limited bioavailability of the active compound (or metabolites) may explain lack of effect at nutritional concentrations.

High-dietary-folate and high-blood-folate status are generally associated with a decreased risk of colorectal cancer and heart disease. Folates have a critical role in maintaining DNA stability by donating 1-C moieties and maintaining DNA methylation. Cell, animal and human studies demonstrate that folate deficiency induces epigenetic changes by attenuating remethylation of SAH to SAM in the methionine cycle, leading to hyperhomocysteinemia, cytosine demethylation, global DNA hypomethylation and proto-oncogene activation. Dysregulated DNA methylation has been proposed to be a common mechanism linking cancer and heart disease by up-regulation of proto-oncogene expression and induction of epithelial (cancer) and SMC (vascular disease) proliferation and migration. However, the effect of folate status on DNA methylation is profoundly dependent not only on the severity and duration of the folate depletion, but also on the gene, tissue and stage of malignant transformation. Similarly, while genome-wide DNA hypomethylation is detected in atherosclerotic plaques from experimental animals and human tissue, the impact of vitamin B and homocysteine on DNA methylation and disease risk

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remains to be established. On balance, the evidence available currently does not strongly support the hypothesis that altered genome-wide DNA methylation, as a direct consequence of low-folate or vitamin B status, increases human heart disease risk.

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