Oncogenic co-operation in β-cell tumorigenesis

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Abstract

Pancreatic islet neoplasms are rare endocrine tumours. The most common type is of β-cell origin and is known as insulinoma, which can be either benign or malignant. The majority of insulinomas arise sporadically, but a small proportion develop as part of the hereditary multiple endocrine neoplasia type 1 (MEN1) syndrome. As for many human tumours, the genetic events that occur during the initiation and progression of insulinoma are poorly known. The men1 gene product, menin, is deficient in most hereditary cases, but is not obviously affected in the majority of sporadic tumours. Activation of the proto-oncogenes c-myc and ras has been observed during malignant progression, but their role in tumour initiation remains unproven. To address these questions, transgenic mouse models have been increasingly used to explore molecular and genetic events that might also precipitate human neoplasia. Transgenic mice expressing SV40 large T-antigen (Tag) oncogene in β-cells develop tumours in a multi-stage progression from hyperplasia, angiogenesis, to solid encapsulated tumours. However, Tag, which inactivates the key tumour suppressors p53 and Rb, is not known to be involved in the pathogenesis of human insulinoma. The proto-oncogene, c-myc is implicated in β-cell growth in both diabetes and tumorigenesis. Activation of Myc appears to be an early event in progression of human insulinoma. The effect of deregulated Myc expression on adult β-cells in vivo has recently been investigated by developing transgenic mouse models in which the activity of Myc can be regulated ectopically. Although Myc activation initially promotes both proliferation and apoptosis in pancreatic β-cells, apoptosis is the predominant outcome, giving rise to islet involution and diabetes. Importantly, inhibiting Myc-induced apoptosis (by co-expression of Bcl-xL) leads to significantly enlarged islets, many becoming highly vascularized, hyperplastic and invasive. These results suggest that, in the pancreatic β-cells, early suppression of apoptosis is essential for the survival of Myc-activated β-cells and islet neoplasia.

Introduction

Insulinomas

Insulinomas are tumours of the islets of Langerhans and may encompass different forms of β-cell disease, all of which produce hyperinsulinism and hypoglycaemia. Although insulinomas are rare (1–5 cases per million per year of population) they are the most common type of pancreatic endocrine neoplasm (Service et al. 1991). The majority of insulinomas arise sporadically, in individuals of a median age of 50 years at diagnosis, and are usually small, single and benign. However, insulinomas arising as part of the hereditary disease, multiple endocrine neoplasia type 1 (MEN1) develop in the mid-20s and are multiple in up to 60% of patients. Most islet tumours remain benign, but a proportion (some 6%) become malignant and are associated with local invasion of surrounding soft tissues or the presence of metastases in liver and local lymph nodes (Broder & Carter 1973). Benign tumours are generally less than 1.5 cm across, whereas malignant tumours are larger. Some tumours secrete several hormones. Not surprisingly, malignant insulinoma has a much poorer prognosis, but even so most patients survive for considerable periods with supportive treatment.

In children, and rarely in adults, a form of diffuse or localised increase in insulin-secreting cells can occur within the pancreas. This so-called nesidioblastosis may co-exist with discrete insulinoma or may occur as a rare autosomal recessive disorder (Fong et al. 1989). In nesidioblastosis, insulin-producing cells differentiate from ductal epithelium and bud from the ducts to form new islets, resulting in an
increase in the total number of islets. New islets vary in size and shape: islet cells can occur singly or in small clusters, and many islets have irregular contours, interdigitating with the adjacent acinar parenchyma. One case report showed that larger, hypervascular islets had a more poorly defined anti-insulin staining pattern than smaller, normal-looking islets (Nathan et al. 1981).

Symptoms of insulinoma primarily reflect hypoglycaemia developing as a consequence of excessive or inappropriate secretion of insulin and proinsulin. Thus patients experience various combinations of visual disturbance, sweating, palpitations, confusion and abnormal behaviour. In fact, up to half of affected individuals may experience episodic unconsciousness and 10–15% will suffer seizures. These symptoms occur primarily during fasting or after alcohol or exercise. Despite the well-known association between hyperinsulinaemia and obesity, only some 20% of patients with insulinoma experience obvious weight gain. Diagnosis is usually made by biochemical analysis rather than by imaging techniques, which in general are used primarily for pre-surgical localization of tumours. Thus the demonstration of inappropriate secretion of insulin and C-peptide, and absence of sulphonylurea drugs supports the diagnosis of insulinoma. However, it may prove necessary to perform a prolonged fast (up to 72 h) in order to provoke hypoglycaemia and thus confirm inappropriate hyperinsulinaemia. Patients frequently have increased proinsulin concentrations and an insulin:proinsulin ratio closer to 1:1 rather than the 6:1 typical of normal individuals.

**MEN1 syndrome**

MEN1 is a hereditary disease associated with increased cellular proliferation in at least two endocrine organs, predominantly pancreatic islets, parathyroid gland, pituitary and adrenal glands (Wong et al. 2000). Insulinoma occurs in 5–10% of patients with MEN1 syndrome. Importantly, many patients also develop tumours in a range of other endocrine and non-endocrine tissues, indicating that the men1 gene product, menin, may have a functional role in a wide variety of tissues. MEN1 is inherited as an autosomal dominant condition. Patients inherit one mutated gene copy and, later in life, a proportion of endocrine cells acquire mutations in the remaining wild-type gene (loss of heterozygosity), leading to elimination of its tumour suppressor activity. Thus, although both gene copies must be lost or inactivated for the expression of a consequent phenotype, the ‘second hit’ will almost inevitably occur in one or more cells within those tissues known to be vulnerable to consequent tumorigenesis. What other mutations may be required for the development of benign or eventually malignant tumours is poorly understood, as is the actual oncogenic sequence involved in the genesis of most human cancers. This situation is compounded, as it is becoming increasingly clear that this sequence is not invariable and that histologically seemingly similar cancer types may arise via differing ‘oncogenic routes’. This is also true of insulinomas. It is interesting to note that, although somatic men1 mutations have been identified in a number of sporadic tumours, including insulinoma (Wang et al. 1998, Wong et al. 2000), a recent study has failed to detect any mutations of the men1 gene in a large number of sporadic insulinomas (24 benign and three malignant) (Cupisti et al. 2000). These apparently contradictory findings may reflect intrinsic variations between the different populations studied. It is likely that this will be resolved only by examining insulinomas in a number of different geographical areas and ethnic groups.

The men1 gene is located on chromosome 11 and encodes a 610 amino acid protein product, menin, which is believed to possess tumour suppressor activity (Chandrasekharappa et al. 1997, Wong et al. 2000). Two transcripts have been identified; most probably they are the result of alternative splicing – a 2.9-kb transcript is expressed in all tissues, and a 4.2-kb transcript in the pancreas and thymus. Menin is predominantly a nuclear protein and specifically binds Jund (a member of the AP1 jun-fos transcription factor family) via its N-terminal and inhibits Jund activation of transcription. However, the true function and importance of the men1 gene in pancreatic islet tumorigenesis has until recently remained unknown. To investigate the role of MEN1 in tumour development, a mouse model was generated by homologous recombination of the mouse men1 gene (Crabtree et al. 2001). Homozygous ‘knockout’ mice were not viable, experiencing developmental delay and embryonic lethality. In these studies 40% of heterozygotes developed large hyperplastic islets between 6 and 9 months of age, but with little evidence of current cell replication at the time points examined. It is not possible to conclude if islet hyperplasia was a product of excess islet cell replication or reduction of apoptosis, as earlier time points were not examined. Evidence of progression to islet tumours was observed in 28% of animals by 22 months and correlated with loss of the wild type allele, with invasion found in only one animal. It is likely that additional, as yet uncharacterized, mutations are required for the formation of tumours in these mouse strains. Although loss of the wild-type allele was detected in the tumours examined, the absence of obvious tumour formation in the majority of animals suggests that this alone may not be sufficient. It will be of interest to assess islet tumorigenesis in adult homozygous conditional ‘knockouts’ when these are available.

**Genetic events in the progression of malignant insulinoma in humans**

Relatively little is known about the genetic events that occur during the initiation and progression of malignant insulinoma...
in humans. However, several observations (Pavelic et al. 1995, 1996) suggest that activation of the proto-oncogenes, c-myc (myc) and ras, and overexpression of transforming growth factor α (TGF-α) and p53 tumour suppressor protein may occur during malignant progression. Immunohistochemical detection of proteins Myc, K-ras and N-ras, TGF-α, and p53 has been performed on archival pancreatic tumour samples. Results have shown that higher levels of Myc are detected in β-cell hyperplastic islets, and benign and malignant insulinomas than in normal pancreatic islets. Ras, TGF-α, and p53 proteins are undetectable in normal islets but weak immunostaining is detected in benign insulinomas, becoming strongly positive in malignant insulinomas. In some patients, activating point mutations in codon 12 of the K-ras oncogene have been detected. Increased concentrations of p53 tumour suppressor protein have been demonstrated in malignant insulinomas. Such changes can result from inactivating mutations leading to increased stability of the p53 protein, though no such p53 mutations have been identified in insulinomas to date. From such observational data a model has been proposed for molecular events that might occur during insulinoma progression: activation of Myc and TGF-α appear as early events, being present at the hyperplastic stage, whereas activating ras mutations and increased concentrations of p53 occur at later stages, in benign and malignant insulinoma.

More recently, an in vitro model of insulinoma has been established (Katic et al. 1999) to determine the effects of myc and ras in pancreatic β-cells. Primary mouse islets were infected with a recombinant retrovirus containing v-H-ras and v-myc oncogenes, in the presence or absence of TGF-α. Whereas wild-type islets grown in culture survive for only 2 weeks, islets transformed with both v-H-ras and v-myc oncogenes became immortal accompanied by loss of differentiation. Importantly, single β-cells liberated from these islets into the surrounding medium gave rise to new islet formation, a situation that is likely to arise in malignant insulinoma in vivo. However, the effects of single oncogenes, myc or ras, on pancreatic β-cells in vitro were not examined. Nevertheless, these results are consistent with a role for myc and ras activation during progression of human insulinoma.

**Suppression of apoptosis in islet cancer**

Apoptosis or programmed cell death is a physiological mode of cell death that occurs during embryogenesis and tissue homeostasis. For example, in the developing nervous system, only half of the original neurones survive, as a result of receiving sufficient survival signals, whereas the remaining cells die by apoptosis. It is likely that similar processes obtain in other tissues, to ensure that cells survive only at the appropriate time and place. Apoptosis continues in many adult tissues throughout life (in an adult human, billions of cells die every hour) and thereby serves to control cell numbers by maintaining a balance between cell proliferation and cell death. Such tissue homeostasis is particularly important in those tissues that have a high cell turnover, for example, epithelial and haemopoietic tissues. As a consequence, when the balance between proliferation and apoptosis is perturbed, profound and deleterious effects on the organism can arise: suppression of apoptosis is essential for tumour development, whereas inappropriate and excessive cell death is associated with degenerative disease such as Alzheimer’s disease and Parkinson’s disease.

Many of the signals that elicit apoptosis converge on the mitochondria, leading to the release of cytochrome C, a potent catalyst of apoptosis (Green & Reed 1998). Members of the Bcl-2 family of proteins possess either pro-apoptotic (Bax, Bak, Bid, Bim) or anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w) function, and act in part by governing mitochondrial death signalling through the release of cytochrome C.

**Islet cell neoplasia in transgenic mice expressing SV40 large T-antigen**

In order to study the events that occur during multi-stage carcinogenesis, a transgenic mouse model was developed in which expression of the SV40 large T antigen (Tag) oncogene is targeted to pancreatic β-cells under the control of the rat insulin promoter (RIP-Tag) (Hanahan 1985, Naik et al. 1996). The Tag oncoprotein exerts its oncogenic effect in part through binding to and inactivating tumour suppressor proteins, pRb and p53. The expression of Tag in RIP-Tag transgenic mice starts at embryonic day 9 and persists in the β-cells of all islets throughout adulthood. All the mice go on to develop a small number of islet cell carcinomas by 14 weeks of age (Hanahan 1985). Tumour progression in these islets proceeds through several stages: 1) 50–75% of islets become hyperplastic (hyperproliferative) containing multi-focal carcinoma in situ lesions by 4–6 weeks of age, 2) 10% of islets become angiogenic by 8–10 weeks of age, 3) 1–2% of islets develop into solid, encapsulated tumours by 11–12 weeks of age.

The switch to hyperplasia coincides with expression of insulin-like growth factor (IGF)-II, which may play a part in inhibiting apoptosis of β-cells, rather than promoting mitogenesis (Christofori et al. 1994). In the transition from normal to hyperplastic, angiogenic, and tumorigenic, normally quiescent islets exhibit increasing mitotic activity (Naik et al. 1996). However, concomitant with proliferation, significant apoptotic death of β-cells (p53-independent in this case) is demonstrable throughout islets at all stages except in solid tumours. Importantly, absence of detectable cell death in Tag-derived islet tumours coincides with an increase in expression of Bcl-xL. In fact, when RIP-Tag mice were subsequently crossed...
Figure 1 Myc-induced proliferation and apoptosis in pancreatic islets. Immunohistochemical staining of tissue sections was performed using Ki-67 antibody, specific for a nuclear protein expressed in proliferating cells, or with TUNEL (TdT-mediated, dUTP nick end labelling) for detection of apoptosis (both brown staining). The pancreatic islets of untreated insulin-mycER mice (‘Myc off’) are identical to those of wild-type animals, with proliferation or apoptosis of β-cells very rarely detected (upper left and upper right panels respectively). Activation of MycER (‘Myc on’) in β-cells of adult transgenic mice induces dramatic proliferation (bottom left panel) and extensive apoptosis (bottom right panel), leading to involution of islets after 7 days of tamoxifen treatment. Size bar represents 50 μm. (Lower left and right photomicrographs reproduced with permission, from Pelengaris et al. 2000.)

with transgenic mice overexpressing Bcl-xL in β-cells, the incidence of apoptosis was greatly reduced in hyperplastic and angiogenic islets, and the incidence of tumour was found to be significantly higher than in RIP-Tag single transgenic littermates (Naik et al. 1996). These results demonstrate that suppression of apoptosis can play a crucial part in the transition from hyperplastic/angiogenic islets to islet cell carcinoma.

Relevance to human islet carcinoma
Although the expression of large T-antigen, as seen in β-cells of Tag transgenic mice, is not known to occur in human islet tumours, the observation that apoptotic regulatory factors (Bcl-xL and IGF-II) are functionally involved in controlling cell death decisions in β-cell tumorigenesis is an important one. For example, increased expression of the anti-apoptotic protein, Bcl-2, has been reported in one-third of human insulinomas (Wang et al. 1997; for review see Wang 1999), suggesting that suppression of apoptosis may contribute to the initiation, progression, or both, of these tumours.
Myc and pancreatic islet neoplasia

Dual potential of Myc – proliferation and apoptosis

The transcription factor c-Myc, encoded by the c-myc proto-oncogene, is a potent inducer both of cell proliferation and of apoptosis in a variety of cell types in vitro (Evan et al. 1992, Amati & Land 1994, Eilers 1999). Recent evidence suggests that c-Myc can sensitize cells to a variety of apoptotic triggers (e.g. DNA damage, serum or growth factor deprivation, hypoxia, tumour necrosis factor (TNF), and CD95/Fas), rather than directly inducing apoptosis by itself (Juin et al. 1999, Prendergast 1999). The pro-apoptotic effect of c-Myc is mediated through release of mitochondrial holocytochrome C (hcC) into the cytosol (Juin et al. 1999). Importantly, this release is inhibited by the survival factor, IGF-1. Subsequently, hcC interacts with Apaf-1 (a mammalian homologue of Caenorhabditis elegans Ced4 adaptor protein) which then recruits and activates pro-caspase 9 (Li et al. 1997). This ternary complex, or ‘apoptosome’, triggers ATP-dependent autocatalytic processing of caspase 9 which, in turn, activates caspase 3 and other effector caspases. Although c-Myc promotes apoptosis by causing the release of hcC, the ability of hcC to activate apoptosis is critically dependent upon other signals (Juin et al. 1999) as mentioned above.

In vitro experiments have demonstrated that the net balance of Myc-induced growth and death may be dictated by the presence of survival signals (Evan et al. 1992, Harrington et al. 1994). It has therefore been suggested that the ability of Myc concomitantly to induce proliferation and sensitize cells to apoptosis may act as a ‘fail-safe’ mechanism, guarding against a single proliferative lesion leading to unrestrained cell growth (Evan & Littlewood 1998). Several conventional transgenic studies using tissue-specific expression of Myc protein have attempted to address this question (Morgenbesser & DePinho 1994). Although much information has derived from such studies, phenotype is difficult to interpret because Myc is expressed throughout development. This is particularly relevant to oncogenes such as myc, which have both growth and death activity and might in themselves also cause genetic instability (Felsher & Bishop 1999b). Tumours arising from sustained oncogene activation arise after prolonged latency and are clonal in origin, suggesting that additional unknown genetic lesions may have occurred to suppress apoptosis. This is consistent with the observed oncogenic synergy between Myc and Bcl-2 in lymphomagenesis (Strasser et al. 1990, 1996).
The notion that Myc-induced apoptosis may act as a ‘fail-safe’ mechanism after Myc activation cannot be tested by constitutive expression of a myc transgene. This limitation has led to the development of transgenic models in which expression of Myc activity is regulated ectopically (Pelengaris et al. 1999, 2000, Felsher & Bishop 1999a). Such studies have shown that Myc is sufficient to induce benign neoplasia and angiogenesis in skin (Pelengaris et al. 1999), but not in pancreatic β-cells (see below). These experiments have demonstrated that the balance between oncogene-induced proliferation and apoptosis in a given tissue can be a critical determinant in the initiation and progression of the tumour (for review see Pelengaris et al. 2000).

**Myc and human pancreatic islet neoplasia**

Myc expression is detected in many human tumours (Spencer & Groudine 1991, Marcu et al. 1992, Nesbit et al. 1999) including those of β-cell origin (Pavelic et al. 1995, 1996, Wang 1997). However, as most data are derived from established tumours or cell lines bearing several oncogenic lesions, it is not known whether expression of Myc is instrumental in the initiation or progression of the neoplastic phenotype, or merely arises as a late event through selection pressures during tumorigenesis.

Factors regulating proliferation and apoptosis in β-cells in vivo remain unknown, but a likely candidate is Myc. For example, islets undergoing hyperplasia in response to hyperglycaemia demonstrate up-regulation of Myc, coincident with a loss of numerous markers associated with β-cell differentiation. Therefore myc is of particular interest in the context of β-cell growth, both in diabetes and in tumorigenesis. Pavelic et al. (1996) proposed a model, as mentioned earlier, outlining molecular events that might occur during human insulinoma progression. Activation of Myc appears as an early event, being present both at the hyperplastic stage and in benign and malignant insulinoma.

**Myc activation causes pancreatic β-cell apoptosis and diabetes in mice**

The effect of de-regulated Myc expression on adult β-cells in vivo has recently been investigated by developing transgenic mouse models in which the activity of Myc can be regulated ectopically. Expression of the regulatable Myc protein, MycER (human c-Myc cDNA fused to the ligand-binding domain of a modified oestrogen receptor; Littlewood et al. 1995) was targeted to β-cells using an insulin promoter. Sustained activation of MycER was achieved by daily intraperitoneal administration of the specific ligand, 4-hydroxytamoxifen (4OHT). Although Myc activation promotes both cell proliferation and apoptosis in pancreatic β-cells in vivo (Fig. 1, lower panel), apoptosis is the predominant outcome, giving rise to islet involution (Fig. 2, middle panel) and diabetes within 7 days (Pelengaris et al. 2000 and unpublished data). These results suggest that, in pancreatic β-cells, early suppression of apoptosis is essential for the survival of Myc-activated β-cells and islet neoplasia.

**Suppression of Myc-induced apoptosis leads to islet carcinoma**

Given the results above, we have proposed a model for Myc-induced tumorigenesis in pancreatic islets involving the balance between proliferation and apoptosis. In pancreatic β-cells, in which the predominant effect of Myc activation is apoptosis, progression to invasive cancer is extremely rare because of efficient elimination of c-Myc-transformed β-cells by apoptosis.

Consistent with this, co-expression of the anti-apoptotic protein, Bcl-xL, inhibits Myc-induced apoptosis and the islets become significantly enlarged (Fig. 2, right panel) as a result of an increase in proliferation and a decrease in cell death (S Pelengaris & G Evan, unpublished data). In fact, after only 1 week of Myc activation, many islets are highly vascularized, hyperplastic and invasive. Clearly, within this tissue, the early suppression of apoptosis is essential for the survival of Myc-deregulated cells and subsequent tumorigenesis.

**Concluding remarks**

Cancer is believed to be a multistage process involving the accumulation of several genetic lesions necessary for the full expression of malignant phenotype. For normal cells to become invasive cancers, they must acquire characteristic properties including self-sufficiency in growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, which may be features of all human cancers (Hanahan & Weinberg 1999). In many established cancers, these properties arise in association with the accumulation of several genetic lesions. However, it is not known whether all such potentially oncogenic lesions are actually required for cancer progression in vivo, or indeed, remain important in tumour maintenance. In fact, for the majority of human cancers the identities of the lesions responsible for the initiation and progression of tumorigenesis are not known. Numerous candidate genes that are de-regulated in established human cancers have been identified. Thus Myc is de-regulated in most cancers, including those of β-cell origin, many of which also over-express the anti-apoptotic protein, Bcl-2. However, as advanced cancers have accumulated numerous mutations, some of which may have no bearing on the tumour phenotype, the functional significance remains unproven.

Such descriptive information must be complemented by prospective data obtained during multistage tumorigenesis. Thus recent advances in the understanding of oncogene co-operation owe much to the development of transgenic
models, which allow the study of tumour development in vivo. Further advances are likely to derive from the increasing use of sophisticated new systems, whereby mouse cancer models can most closely reproduce the likely events occurring in their human counterparts. Thus, regulatable transgenic models allow the expression or activation of oncogenes, such as Ras and c-Myc, in distinct adult tissues rather than throughout ontogeny. Related systems are being applied to regulate the activity of Cre-recombinase in order that specific genes (such as tumour suppressor genes) can be ‘knocked-out’ in adult tissues. This technology will make it possible to investigate the effects of targeted expression or disruption of specific genes implicated in cancer within relevant adult tissues. Furthermore, the potential subsequently to reverse such genetic lesions may have therapeutic implications in the identification of potential treatment targets.

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