Analysis of Wnt/Beta catenin signalling in desmoid tumors

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Abstract

Desmoid tumors are fibromatous lesions occurring both sporadically and in patients with familial adenomatous polyposis (FAP). Because of the association of these tumors with the hereditary colorectal cancer syndrome FAP we set out to define the molecular events driving desmoid tumorigenesis, hypothezising these might be identical to events driving colorectal tumorigenesis. We found that whereas FAP-associated desmoid tumors are caused by germline APC mutations followed by somatic inactivation of the wild-type APC allele, sporadic desmoids are usually characterized by oncogenic mutations in the b-catenin gene, both identical molecular alterations to those found in the vast majority of colorectal cancers. Next we set out to investigate the cellular pathways activated by these mutations, and identified activation of the Wnt signaling pathway in desmoid tumors. Wnt signaling modulates expression of developmental genes and cell fate via ß-catenin, and has been implicated in many cancer types. Currently we are investigating tissue-specific downstream effectors of the Wnt pathway that might be responsible for the behaviour of these invasive fibrous tumors. Our findings also point to a role for this pathway in the regulation of normal myofibroblast proliferation and suggest novel treatments in desmoid tumors and other fibrous proliferative disorders. (Acta gastroenterol. belg., **2005**, 68, **5-9**).

Introduction

Biological processes like embryogenesis and carcinogenesis, although seemingly distinct, both rely on cell communication via identical signaling pathways. The clues to understanding cancer often rely on the knowledge of the normal regulation of its tissue of origin. This concept is well developed in the field of cancer research, where it was discovered that many oncogenic mutations activate developmental signaling pathways. Our research focuses on the dysregulation of Wnt/ß-catenin signaling in desmoid tumors. Desmoid tumors are benign fibrous lesions consisting of an unchecked proliferation of fibrocytic cells. The Wnt/ß-catenin pathway is a receptor-mediated intracellular signal transduction pathway conserved during evolution in both invertebrates and vertebrates. Wnt signaling modulates expression of developmental genes and cell fate via ß-catenin often in a combinatorial manner with other pathways. During the course of our work on the molecular alterations in desmoid tumors we found a constitutive reactivation of Wnt/ß-catenin signaling in these tumors. This finding may point to a role for this pathway in the regulation of normal fibroblast proliferation and suggest novel treatments in desmoid tumors and other pathologic conditions. In addition, the implication of ß-catenin signaling in an oncogenic process provides us with a little more insight into the multiple roles of this fascinating protein.

1. *Multiple roles of ß-catenin : integration of cell adhesion with gene expression*

ß-catenin was initially discovered in1989 as a component of the adherens junction (1). It soon became apparent that ß-catenin can play different roles in the cell, one as a structural protein at cell-cell adherens junctions and another as a transcriptional activator mediating Wnt signal transduction. Cell-cell adhesion is mediated by a family of molecules called cadherins, which are calcium dependent-transmembrane glycoproteins. The dogma of cell adhesion is that the cadherin cytoplasmic domain binds to ß-catenin or plakoglobin, which binds to α-catenin, which in turn connects the complex to the cytoskeleton. Tyrosine phosphorylation of ß-catenin appears to be a key step in the dissociation of the cadherin cytoskeleton interaction. Increased tyrosine phosphorylation of ß-catenin results in its dissociation from the cadherin complex and an increase in the free cytoplasmic pool of ß-catenin.

The work reported here on the cellular effects of stabilized ß-catenin in desmoids has focused on its signaling properties, but further experiments are ongoing to study a potential cadherin-mediated role in the phenotype of these tumors.

2. *The Wnt signaling pathway*

The development of a fertilized egg into a multicellular organism requires coordination of many processes. Each cell must choose the proper cell fate and must also assume its place as part of an organized tissue. One conserved pathway that directs cell fate decisions in many animals is the Wingless (Wg)/Wnt signal transduction pathway. Together with other families of secreted factors such as fibroblast growth factor (FGF), transforming growth factor β (TGFβ) and Hedgehog proteins, Wnt proteins are implicated in a wide variety of biological processes. During normal development, most cells do

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not receive Wnt signals. When Wnt is absent, ß-catenin is degraded and levels of cytoplasmic ß-catenin are very low. ß-catenin degradation occurs by the formation of a cytoplasmic degradation complex containing at least axin, APC, Glycogen synthase kinase-3ß (GSK3ß) and ß-catenin, which leads to the phosphorylation of ß-catenin and axin by GSK3ß. This promotes interaction of ß-catenin with ß-TrCP, leading to the ubiquitination of ß-catenin and its degradation by the proteasome. However, Wnt signal relieves the destruction of ß-catenin. ß-catenin accumulates, translocates into the nucleus, and binds LEF/TCF, forming a bipartite transcription factor that turns on Wnt-responsive genes. The Wnt/ß-catenin pathway is the best understood Wnt pathway but other non canonical Wnt signaling pathways exist in vertebrates and invertebrates (2). Our research in desmoid tumors identified abnormal ß-catenin-mediated signaling, and for this reason we will limit our discussion of the Wnt pathway mainly to ß-catenin mediated effects.

3. *ß-catenin as an oncogene*

Because activation of the Wnt pathway stimulates cell growth, researchers had long suspected that too much Wnt signaling could cause problems. The subsequent elucidation of the Wnt signaling pathway led to the discovery of many cancer causing genes in this pathway. Three regulatory genes (APC, ß-catenin and Axin ; 3) in this pathway are known to be mutated in primary human cancers and several other genes in this pathway promote experimental cancers in rodents. Elevated levels of ß-catenin are found in cancers of various origins (4). These increases in ß-catenin levels result mostly from mutations in ß-catenin itself that affect residues in the GSK3ß phosphorylation sites critical for ß-catenin degradation. In addition, mutations in key components of the degradation machinery, such as APC or axin, have also been detected in some tumors (5).

4. *Desmoid tumors*

Fibromatoses cover a broad group of benign fibrous tissue proliferations of similar microscopic appearance that are intermediate in their biological behavior between benign fibrous lesions and fibrosarcoma. Histologically, desmoid tumors are defined by well-differentiated intertwining fibroblasts and myofibroblasts arranged in broad bundles (6). The cells are slender and spindle shaped, of uniform appearance, and separated from one another by abundant wavy collagen (pro-α1I collagen and pro-α1 III collagen). The putative cell of origin of desmoid tumors, remains an elusive cell. Several cells might serve as progenitors of myofibroblasts : the fibroblast, the pericyte, the vascular smooth muscle cell, and all stromal cells with myoid features. In turn, these cells might all represent cellular isoforms of a common ancestor cell, which when stimulated by environmental factors, might transform into myofibroblasts according to functional demand. One of our current research areas is identifying the precursor cell of desmoids, and we are focusing on the mesenchymal stem cells. Myofibroblasts are also found in granulation tissue of normal healing wounds. This could be particularly relevant for the etiopathogenesis of desmoid tumors, since desmoid cells closely resemble these myofibroblasts. The proliferation of desmoid cells seems to be triggered by cytokines, as in normal wound healing, but defects in the negative feed-back loop (through molecular alterations) might account for their continuing proliferation. Interestingly, we and others have found the main characteristic of desmoid tumors, stabilized beta catenin, to be present during the proliferative phase of wound healing.

The etiologic factors causing desmoid tumors have not been well defined. They frequently appear to be associated with trauma, either surgical or physiologic and radiation. The availability of a FAP mouse model for desmoid tumors (7) will allow further investigation of the etiologic factors in desmoid tumors, such as surgically inflicted scars, inflammation and hormones.

Cytogenetic and molecular studies (8) have revealed desmoids to be clonal, neoplastic proliferations. Cytogenetic studies in fibromatosis have revealed some non-random chromosomal abnormalities such as trisomy 8 and trisomy 20 (9). Structural changes may also occur and especially changes involving the long arm of chromosome 5. Since the APC gene was mapped to the long arm of chromosome 5, 5q 21-22 (10), several authors have proposed involvement of this gene in the development of desmoids. Sporadic desmoids are quite rare and have been estimated to occur in 2 to 5% per million population per year, whereas they occur in about 10% of FAP patients (11), further suggesting a role for APC.

5. *Evidence of APC involvement in desmoid tumors*

Desmoid tumors occur as sporadic lesions or as part of the familial conditions Familial Adenomatous Polyposis (FAP, OMIM 175100). FAP is an autosomal dominantly inherited disorder characterized by the development of hundreds to thousands of adenomatous polyps of the colon. Extracolonic manifestations of FAP include duodenal adenomas, gastric fundic cystic glands, osteomata, epidermoid cysts, congenital hypertrophy of the retinal pigment epithelium and desmoid tumors. The syndrome is caused by germ-line mutations in the APC gene (12). Colorectal polyps and tumors in FAP patients show a biallelic inactivation of the APC gene, one allele carrying the inactivating germline mutation, and the other allele being affected by a somatic mutation. This is in keeping with the role of APC as a tumor suppressor gene in colorectal cancer (through its negative regulation of ß-catenin).

Our aim was to determine the prevalence and type of APC gene mutations in sporadic desmoid tumors.

Somatic APC gene mutations were found in 5 out of 34 sporadic desmoids (15%). The second allele was lost by deletion in 4 tumors and inactivated by a somatic mutation in one. Biallelic inactivation of the APC gene in desmoid tumors is consistent with a tumor suppressor function of APC in these cells. Interestingly, all mutations were clustered within a 111bp region of the APC gene (4% of the coding region). This region represents a mutational hotspot specific for desmoid tumors leading to a characteristic truncated protein. In our series we observed a mutation cluster region in desmoids different from the mutation cluster in colorectal cancer. This desmoid mutation cluster region (d-MCR) is located more 3' than the MCR, roughly codons 1445 to 1580.

6. *ß-catenin mutations in sporadic desmoid tumors*

The presence of mutations involving both alleles of the APC gene in a subset of sporadic desmoid tumors suggests a role for this gene in the pathogenesis of these tumors. APC is necessary for efficient ß-catenin degradation and these mutations lead to the accumulation of ß-catenin in the tumor cells. Since only a small percentage of desmoids carried APC mutations, mutation analysis on ß-catenin itself was performed. ß-catenin contains 4 N terminal serine/threonine residues, which are phosphorylated by GSK3ß, when ß-catenin is bound by APC and Axin. Phosphorylation by GSK3ß of ß-catenin at these specific amino terminal sites targets ß-catenin for degradation. Twenty-two of forty-two samples analyzed demonstrated mutations in ß-catenin (13). Ten resulted in a substitution of alanine for threonine at codon 41 and twelve resulted in a substitution of phenylalanine for serine at codon 45. Beta catenin mutations were exclusive to those that inactivate APC. This exclusivity of ß-catenin and APC mutations was previously demonstrated in colorectal cancer (14), where the vast majority of tumors contain APC mutations and the overall frequency of ß-catenin mutations is quite low. But when colorectal tumors with microsatellite instability (due to defects in DNA mismatch repair) are analyzed separately, the likelihood of finding a ß-catenin mutation is greatly increased. We however found no indications of microsatellite instability in ß-catenin mutant desmoid tumors. A total of 22 mutations in ß-catenin and 9 mutations in APC were identified in this series of sporadic desmoid tumors. Taken together, about 75% of tumors thus had identified mutations, whereas 25% remain as yet unexplained. Immunohistochemical analysis of ß-catenin expression however, revealed increased ß-catenin protein expression in all 42 lesions, regardless of their mutational status. This suggests that other genes acting in the ß-catenin degradation complex could be mutated in these remaining tumors.

7. *ß-catenin protein stabilization in desmoid tumors*

The APC mutations identified in desmoid tumors are predicted to lead to an inefficient degradation complex, hence ß-catenin protein stabilization. The mutations identified in ß-catenin itself, abolish the phosphorylation sites necessary for the recognition by the degradation machinery, thus leading to ß-catenin protein stabilization. An immunohistochemical analysis of the ß-catenin protein was performed. ß-catenin protein expression was increased as compared to normal marginal fascia in all lesions studied, regardless of their clinical, karyotypic or mutational characteristic.

Tumor tissue showed intense staining in the cytoplasm and the nucleus of most tumor cells. ß-catenin was also found in almost all the normal fibrocytes, although staining here localized to the cytoplasm or the cell membrane. Western blotting after cell fractionation showed accumulation of ß-catenin in the cytoplasmic and membranous cell fraction in the tumors, whereas in normal fibroblasts ß-catenin was only found in association with the cell membrane The localization of ß-catenin in the cell nucleus supports its possible function as a regulator of transcription in these tumors.

8. *ß-catenin-LEF/TCF mediated transcriptional activation in desmoid tumors*

Stabilized ß-catenin can bind transcription factors of the LEF/TCF family, translocate to the nucleus, and transactivate transcription. LEF/TCF family members are architectural transcription factors, whose ability to transactivate transcription is altered by binding to ß-catenin. The result of this stabilization may vary between tumor types, as colonic polyps can go on to malignancy, while desmoid tumors are only locally invasive. One possibility is that stabilized ß-catenin binds different transcription factors in these lesions, which will alter transcription in a different manner. Binding to different transcription factors is already demonstrated between colonic neoplasia (in which ß-catenin binds TCF-4) and pilomatricomas (in which ß-catenin binds LEF-1). Thus, it is possible that different transcription factors, or other cell type specific factors, are responsible for the difference in behavior between lesions such as colonic polyps and desmoid tumors. Thus, we set out to identify the LEF/TCF transcription factors that are expressed in fibromatosis lesions. Only TCF-3 was consistently expressed in desmoid tumors. TCF-4 was expressed in only three of the ten aggressive fibromatoses, while TCF-1 and LEF-1 were not expressed. This is in contrast to colon cancers, which express TCF-4 and TCF-1. Normal fibrous tissues from the ten aggressive fibromatosis patients (fascia from the resection margin) all expressed TCF-3 using RT-PCR, but did not express any of the other transcription factors (15).

After identification of LEF/TCF transcription factors expressed in desmoids, we wanted to test for the presence of constitutive ß-catenin/TCF transcriptional activity in the tumors. For this we transfected pTOPFLASH TCF reporter into primary tumor cell cultures. The pTOPFLASH reporter construct contains copies of the

consensus binding sequence for TCF transcription factors linked to a luciferase reporter and the pFOPFLASH reporter construct contains a mutated binding sequence linked to the reporter as a control.

Desmoid tumors primary cell cultures show constitutive TCF transcriptional activation, as demonstrated by a significant increase in the ratio of luminescence between the active reporter construct (pTOPFLASH) and the mutant reporter construct (pFOPFLASH) over that observed in primary cell cultures from normal fibrous tissues from the same patients (ratio of 12.5-vs-2.5, $p < 0.05$). Using specific TCF antibodies we performed additional co-immunoprecipitation experiments. Co-immunoprecipitation using TCF-1, TCF-4, and TCF3/4 antibodies was used to determine if ß-catenin binds the TCF transcription factors in desmoid tumors. We found that ß-catenin co-immunoprecipitated with TCF-3 in the desmoid tumors, but not with TCF-4. This is in contrast to immunoprecipitations from SW480 cell line extracts, in which TCF-4 immunoprecipitates contained ß-catenin protein.

9. *Differential gene expression analysis*

Our previous results showed that ß-catenin-mediated TCF-dependent transcription was activated in fibroblasts of desmoid tumors. We hypothesized that genes regulated by ß-catenin mediated TCF dependent transcription are at least partially responsible for the cell behavior in these tumors. A large number of genes contain TCF sites in their promoter sequences and can be differentially regulated by TCF dependent transcription. Since the TCF transcription factors are architectural factors, in many cases, either their activation will enhance or inhibit the expression of genes, whose regulation is primarily controlled by other transcription factors. Furthermore, the genes regulated by TCF activation are likely to be cell type specific. Although there are other studies of genes regulated by TCF-dependent transcriptional activation in epithelial cells (colorectal cancer cell-lines, myc ; cyclin D1), these results do not necessarily translate to other cell types. The initial experiments reported here were performed in primary desmoid cultures and primary fibroblast (fascia) cultures of the same patient. Oligonucleotide arrays (Affymetrix) were used to identify differentially expressed cDNA's. 69 genes were identified as being significantly differentially expressed, of which about half were upregulated in desmoids and the others significantly downregulated when compared to fascia. Except for MMP-7, no known beta catenin target genes in colorectal cancer were differentially expressed. A subset of genes was validated by additional methods such as Northern blot and quantitative PCR. The results of the microarray analysis proved very robust, but only reflect differences in expression between desmoid and fascia, and do not necessarily contain direct beta catenin target genes. Some genes were selected for further analysis such as IGFBP6, a gene downrgeulated in desmoids. IGFBP-6 is active inhibitor of IGFII activity. Promotor analysis showed that stable beta catenin suppressed IGFBP6 poromotor activity, and that this repression was mediated by two TCF consensus binding sites (16). This was the first report of a gene downregulated directly by beta catenin, and further experiments are ongoing to elucidate the mechanism of this repression. Another group of genes that were analysed are members of the MMP family. These were clearly overexpressed in desmoids. Functional assays were performed to analyse their role in the invasive phenotype of desmoids. Collagen and matrigel invasion test demonstrated the presence of invasion stimulating soluble factors, produced by the desmoids, and implicated the MMP's as one such soluble factor (17). Other genes are currently being investigated in our lab (18). Further study of these genes will give novel insights into how TCF dependent transcription modulates cell behavior, and will identify novel therapeutic targets for neoplasia and disorders of wound healing.

10. *Summary and future perspectives*

Our group has shown that sporadic desmoid tumors all contain elevated levels of beta catenin and that this is due to somatic mutations in either the APC gene or in the beta-catenin gene occurring in the tumor cells. APC and beta-catenin belong to an important signalling pathway, implicated in different processes of embryonic development and homeostasis of some adult tissues. Wnt signalling results in intracellular stabilized beta catenin, that cooperates with transcription factors of the Lef/Tcf family to transcriptionally regulate specific target genes. Apc is involved in the degradation of beta catenin and thus downregulation of wnt signalling. Loss of function of APC or activating mutations of beta catenin results in constitutive activation of the pathway. A deregulation of the Wnt signalling cascade is thought to be the cause of colorectal cancer, by aberrant reactivation of Wnt-tcf4 signalling found in and central to the homeosatasis of the stem cell compartment of the colon. Similarly, in desmoid tumors the mutations found in APC and beta catenin lead to the stabilization of the beta catenin protein and constitutive signaling activity. A mouse model for multifocal desmoid disease, Apc1638N, has been generated by targeting the 3' end of the mouse Apc gene. Heterozygous Apc+/1638N animals develop an average of 30 individual desmoids per animal. Using transgenic mice expressing conditional stabilized ß-catenin, it was recently demonstrated that ß-catenin stabilization in fibroblasts is sufficient to cause aggressive fibromatosis (19). Our group demonstrated that constitutive ß-catenin/TCF mediated transcription was activated in desmoids. This suggested that uncontrolled transcriptional activation of this developmental signaling pathway might induce inappropriate proliferation of fibroblasts and thereby induce desmoids.

Recent data from our group and others in wound healing, suggest an even more widespread role for Wnt/β-catenin signaling in fibrous proliferation. In non tumoral myofibroblasts of wounds, it has been suggested that beta-catenin-mediated signaling is activated downstream of growth factors released during the initial phase of wound repair (20). This lead us to the hypothesis that the Wnt signaling pathway controls fundamental processes in myofibroblast proliferation, differentiation and death. Desmoid tumors, as a monoclonal poulation of beta-catenin mutant fibrocytes, constitute a powerful model to dissect the role of wnt/β-catenin signaling in this cell type.

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