INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

RELEVANCE OF CD44 TO CANCER BIOLOGY

Zbigniew Rudzki

Department of Pathology

McGill University, Montreal

April, 1997

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science.

TABLE OF CONTENTS

ABSTRACT

This thesis explores the role of an adhesion protein, CD44, in the biology of colon cancer. CD44 glycoprotein is a main extracellular receptor for hyaluronic acid. A single CD44 gene composed of 20 exons encodes a variety of isoforms due to alternative splicing of 10 middle exons. Overexpression of CD44 and appearance of abnormal isoforms containing products of variant exons have been implicated in the origin and progression of cancer, including human colon carcinoma.

Of two main parts comprising the dissertation, the first one (*"CD44 and the adhesion of neoplastic cells"* by Z. Rudzki and S. Jothy) reviews the current state of knowledge on CD44, emphasizing its role in neoplastic processes. The second part (*"Changes in CD44 expression during carcinogenesis of the mouse colon"* by Z. Rudzki, L. LeDuy and S. Jothy) reports an experimental study of CD44 expression in a murine model of colon cancer. The tumors were induced by subcutaneous injections of a colon - specific carcinogen (1,2-dimethylhydrazine). CD44 expression was studied by RT-PCR/Southern blot and immunohistochemistry. The CD44 transcripts were generally strongly overexpressed in tumors compared with normal colon. Both neoplastic and normal colon samples exhibited the same complex array of transcript bands representing the standard molecule (CD44s) and its variant isoforms. Immunohistochemistry revealed marked heterogeneity of tumor staining, contrasting with a rather uniform mRNA overexpression. There was a significant tendency towards the progressive loss of CD44 immunoreactivity in larger and invading tumors. It is concluded that CD44 isoforms are globally overexpressed at an early, premacroscopic stage of colonic carcinogenesis. Expression of CD44 in the murine model of colon cancer shows some similarities to its human counterpart.

Cette thèse explore le rôle de CD44, une protéine d'adhésion, dans la biologie du cancer du colon. CD44 est une glycoprotéine dont la partie extracellulaire est un récepteur pour l'acide hyaluronique. Le gène de CD44 est composé de 20 exons codant une multitude d'isoformes générées par épissage alternatif de 10 exons intermédiaires. La surexpression de CD44 et la présence d'isoformes anormales contenant des produits d'exons variants ont été impliquées dans l'origine et la progression du cancer, y compris le cancer du colon humain.

Cette thèse comprends 2 parties. La première ("*CD44 and the adhesion of neoplastic cells*", Z. Rudzki and S. Jothy) est une revue de l'état actuel des connaissances concernant CD44, focalisée sur son rôle dans le processus néoplasique. La deuxième partie ("*Changes in CD44 expression during carcinogenesis of the mouse colon*", Z. Rudzki, L. LeDuy, and S. Jothy) décrit une étude expérimentale concernant l'expression de CD44 dans un modèle murin de cancer du colon. Les tumeurs ont été induites par injections sous-cutanées d'un carcinogène spécifique du colon, la 1,2-diméthylhydrazine. L'expression de CD44 a été étudiée par RT-PCR/buvardage Southern, et par immunohistochimie. Les transcrits de CD44 sont globalement surexprimés dans les tumeurs par rapport au colon normal. Les néoplasmes et les colons normaux expriment le même motif de bandes de transcrits représentant le CD44 standard (CD44s) et ses isoformes variantes. L'immunohistochimie montre une hétérogénéité dans l'immunocoloration, contrastant avec l'uniformité relative de la surexpression de l'ARN messager. Une perte progressive de l'immunoréactivité de CD44 a été observée dans les tumeurs volumineuses et envahissantes. Il est conclus que les isoformes de CD44 sont globalement surexprimées à un stade précoce, prémacroscopique dans la carcinogenèse du colon. L'expression de CD44 dans le modèle murin du cancer du colon possède certaines similitudes avec sa contrepartie humaine.

INTRODUCTION

Colorectal cancer: epidemiology, etiology and pathogenesis, and pathology.

Colorectal cancer, a malignant neoplasm originating from the epithelial lining of the large intestine, belongs to one of the few unfavorable aspects of life in contemporary affluent societies. An individual living in the United States, Canada, Western Europe or Australia has approximately a 5% life-time risk of developing this disease, with small variations due to sex, race, country of origin and socioeconomical status.[1] However, even in the countries in which colorectal carcinoma is relatively rare, such as India or Colombia, it is by no means exceptional, since its incidence oscillates around five to ten cases/100,000 people/year.[2] Over the last several decades, the incidence of colorectal cancer in high risk countries has remained almost stable. At the same time countries belonging previously to the low or middle risk group, such as Poland or Japan, have experienced a marked increase in the number of cases, which was paralleled by progressive "Westernization" of their local diet.[1] Immigrants, and especially their children, moving from areas in which colorectal cancer is uncommon, to the Western countries, gradually acquire the risk typical for the native population, provided their acculturation includes adaptation of local dietary habits.[2] Taken together, these epidemiological data strongly suggest that dietary factors contribute to development of colorectal carcinoma. The complexity of a human diet precludes the unequivocal and exhaustive identification of all carcinogenic or protective components of an everyday meal. The simple hypothesis proposed by Burkitt over 25 years ago[3], linking low fiber consumption with high risk of colon cancer risk, is still the best available explanation of up to tenfold differences in incidence between different populations. It is also corroborated by studies of animal models.[4,5] Total dietary fat is generally believed to contribute to higher risk of colon, but not rectal cancer.[2] The precise composition of dietary fat is probably important, since comparison of total per caput fat consumption in various countries reveals sometimes surprising facts (e.g. almost identical consumption in moderate risk Poland and high risk Australia, or strikingly high fat intake in Denmark, not paralleled by exuberant colon cancer incidence among Danes).[2]

Dietary habits, representing environmental risk factors, are responsible for the majority, but not all cases of colorectal cancer, traditionally referred to as "sporadic" cases. As opposed to cuisine trends, the genetic predispositions are much less prone to rational human manipulation. It is already well known that some people, if left untreated (and only potentially effective treatment means often the total colectomy), are virtually destined to develop a colon or rectal cancer. Additionally some other individuals, although not totally doomed, have their risk far above the average. The two most important hereditary syndromes, associated with an unusually high incidence of colorectal cancer, are familial adenomatous polyposis (FAP), called also adenomatous polyposis coli (APC) and hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome). Investigation of the molecular biology characteristics of these syndromes contributed also to the understanding of the genetic mechanisms leading to the occurrence of the more common sporadic cases.

FAP, accounting currently for fewer than 0.2 % of colorectal cancers, results from a germline mutation in the tumor suppressor APC, and is inherited as an autosomal dominant trait, expressed phenotypically in about 1 in 7000 individuals, with nearly complete penetrance.[6] In typical, untreated cases at least one of many thousand of tubular adenomas, carpeting the whole large intestine of an affected individual, progresses to carcinoma at the median age of 42 years, which is at least two decades earlier than the peak incidence for sporadic tumors.[7] Although it was widely believed that the adenomatous proliferation of colonic epithelial cells in FAP was exclusively caused by somatic inactivation of the second copy of APC gene (usually by means of allelic loss), it is currently known that the mutant APC can also exert a dominant negative effect.[8] The adenomas found in FAP do not progress to cancer more frequently or rapidly than those found in nearly half of the general population, but it is the very number of them which makes malignant transformation in FAP unavoidable.[7] In an overwhelming majority of cases the germline mutation (frameshift, splicing or nonsense) leads to the formation of a truncated APC protein, detectable using the *in vitro* translation assay.[9] The exact location

of mutation within the APC gene profoundly influences the colonic and extracolonic manifestations of FAP[6], which can present as:

- a classical form (several hundred to several thousand of colonic polyps with relative sparing of rectum and cecum),
- accompanied by CHRPE (congenital hypertrophy of the retinal pigment epithelium),
- associated with retroperitoneal or abdominal desmoid tumors (often very aggressive and more difficult to control than the polyposis),
- an attenuated phenotype, with few flat polyps, potentially confused with other polyposis syndromes,
- profuse polyposis, with early onset and numerous digestive tract polyps, involving not only the colon.

FAP associated with numerous possible extracolonic manifestations is sometimes referred to as Gardner's syndrome, originally described as a combination of intestinal polyps, osteomas, fibromas and epithelial cysts in a Utah Mormon family.[10]

Tumors arising in FAP develop through the well characterized sequence of at least seven mutations involving APC, K-RAS, oncogenes at 18q21 (DCC, DPC4, JV18-1 and possibly others) and p53, which is paralleled by morphologic progression from normal epithelium to invasive carcinoma through stages such as dysplastic aberrant crypt foci, adenoma with increasing dysplasia and carcinoma in situ.[11] Very similar genetic and morphologic multistep processes take place during the development of the majority of sporadic colorectal cancers, with the only exception that mutations of both APC alleles are somatic in typical sporadic cases.[12]

HNPCC, the second relatively common syndrome linked with the inherited predisposition to colorectal cancer, is caused by a germline mutation of one of the genes responsible for repairing of DNA mismatches and accounts for 2-4% of colorectal cancer cases.[13] The patients do not develop adenomatous polyps more frequently than the general population, but the few "statistically expected" adenomas progress almost invariably to carcinoma, usually in the early fifth decade of life. Extracolonic malignancies, most

notably those of endometrium, ovary, stomach, small intestine, ureter and renal pelvis are found in some pedigrees or individuals.[13] The genetic hallmark of the syndrome, the widespread instability of short repetitive DNA sequences (microsatellites), can be relatively easily detected in almost all tumors in HNPCC. Mutations typical for "APC pathway" (like those of APC, K-RAS, DCC, or p53) are rare in Lynch syndrome.[12] Specifically affected genes identified to date, such as TGF-ß type II receptor, BAX, hMSH6 DNA mismatch repair gene or IGF type II receptor, contain microsatellite sequences within their coding or controlling regions.[14 15] Similarly to FAP, also the HNPCC constitutes a model of genetic development of some sporadic cancers. Approximately 13% of sporadic colorectal carcinomas show mutation pattern typical for mismatch repair deficiency/ microsatellite instability instead of more frequent APC/K-RAS/p53 pathway.[7]

Apart from HNPCC and FAP, the colon (but not rectal) carcinoma cases tend to cluster in some families, with an increased risk of 1.65 - 2.1 for the first degree relatives of an affected individual.[16 17] It is estimated that approximately 50% of colon tumors arise from some kind of inherited susceptibility and that the yet unknown predisposing gene(s) can probably occur in one-third of the population.[16] Additionally, numerous rare, either inherited or nonfamilial syndromes (Peutz-Jeghers, Muir-Torre, juvenile polyposis, hyperplastic polyposis, two variants of Turcot's syndrome to name the few) increase the risk of malignant transformation in the colon, but not necessarily originating from the polyps.[18] Together they account for less than 1% of all cases of colorectal carcinoma.

Colorectal cancer is roughly 20 times more frequent in both ulcerative colitis and Crohn's disease, with an incidence strongly related to the anatomical extent and to the duration of the disease.[19] Other well-documented risk factors predisposing to colorectal carcinogenesis include previous irradiation of the pelvis and obesity.[2 20]

The morphologic characteristics of colorectal carcinoma are much less diverse than its etiology. Most colorectal cancers, regardless of their genetic background, present as either bulky polypoid masses or infiltrative, ulcerating lesions, sometimes with annular constriction of the entire intestinal circumference.[21] The former pattern is more common,

but not exclusive, in the right colon, whereas the latter predominates on the left side. Microscopically over 80% of colonic carcinomas are conventional (gland-forming) adenocarcinomas, 10% are classified as mucinous, 1% as signet-ring cell adenocarcinomas and the remaining group as rare variants.[1] Carcinomas arising in the setting of HNPCC and their sporadic mismatch deficient analogues are more frequently right-sided, poorly differentiated, with a mucinous or signet ring-cell histology, surrounded by a florid lymphoid reaction.[13]

The survival of colorectal cancer patients is determined mainly by the stage of their disease, usually classified according to Dukes' system or its numerous modifications.[1] Grade of differentiation constitutes the only other unquestionably accepted independent prognostic parameter.[1] Five-year survival figures vary significantly between countries (e.g. below 30% for Cracow, Poland, versus around 50% for the USA)[2,22], which probably reflects mainly the differences in the extent of prophylactic screening tests and in quality of therapy.

Rationale of a search for new markers of malignant potential of colorectal cancer.

Colon cancer has at least two unique features distinguishing it from malignant neoplasms responsible for substantial morbidity and mortality. First, it is usually symptomatic at the relatively early stage of its progression, often before metastatic spread takes place. Second, at least 50% of cases involve identifiable high-risk individuals, so that costly screening procedures can be limited to a preselected segment of population. Due to these properties 75% of patients are diagnosed with a potentially resectable tumor (Dukes stage A-C). Several biological characteristics of colon cancer make it an almost ideal candidate for curative surgical resection. The colon has distinct anatomical boundaries with a thick muscle layer forming a barrier temporarily capable of withstanding neoplastic invasion. Although colon cancer first invades the lamina propria, intramucosal carcinomas never metastasize, in comparison with similarly advanced gastric tumors that, at the same

stage, occasionally spread to the regional lymph nodes.[20] The reason for this relative indolence of early colon cancer is not entirely clear since colonic lamina propria contains some lymphatics at least in its lower portion.[20,23] Compared with gastric or pancreatic malignancies, in most cases a surgeon can define the extent of a colon tumor by palpation and specify safe resection margins. Colon is one of the barriers between the precisely regulated and protected interior of human body and the harsh, unpredictable external environment. Hence it is well guarded by checkpoints that includes lymph nodes which can intercept not only microbial pathogens but also metastatic cancer cells. Due to limited anatomical variability and their intramesenteric location, these lymph nodes are easily excised together with a colectomy specimen. Direct blood-borne spread of neoplastic cells with bypassing of the lymph node barricade is exceptional. Finally, most colon cancers grow relatively slowly and are well or moderately differentiated malignant neoplasms.

Despite these seemingly favorable clinical and biological features of colon cancer, approximately 1/3 of patients diagnosed with a potentially curable neoplasm develop recurrences after surgery and die of their tumors.[24] Current diagnostic procedures do not allow the separation of this group with an acceptable specificity and sensitivity until therapeutic failure is evident. Therefore, successful identification of new markers and diagnostic parameters could result in more aggressive therapeutic approach and more stringent post-therapeutic monitoring of affected patients. Adhesion molecules are promising candidates among a vast group of prospective markers.

Adhesion molecules and their involvement in tumorigenesis.

Presently known cell surface adhesion molecules can be grouped into five families of proteins: cadherins, integrins, members of the immunoglobulin supergene family, selectins and CD44.[25]

Cadherins are transmembrane molecules with their extracellular portion folded into five similar domains, and an intracellular tail capable of binding to actin via the family of catenins.[26] In the presence of calcium ions, they mediate very strong interactions with the

same kind of molecule on another cell, whereas in the absence of calcium they are rapidly degraded leading to disruption of cell adhesion.[26]

The vast family of integrins is composed of heterodimeric proteins functioning as cell-cell adhesion molecules, cell-substrate adhesion molecules or receptors for extracellular ligands.[27] Some integrins bind extracellular matrix components such as fibronectin, laminin, collagens and osteopontin, thereby facilitating cell migration.[27] Other integrins act as cell adhesion molecules (CAMs). These integrins usually bind to members of the immunoglobulin supergene family.

The members of the immunoglobulin supergene family of proteins probably evolved from an ancestral gene coding for a single Ig-like domain, forming a ß-pleated sheet and stabilized by a disulfide bridge.[28] Currently this family contains numerous molecules sharing various numbers of this basic unit and fulfilling diverse functions. Some of its members, such as neural cell adhesion molecule (NCAM), vascular cell adhesion molecule (VCAM) or carcinoembryonic antigen (CEA) are implicated in homotypic, calcium-independent cell-cell interactions.[25,29]

The selectins recognize fucosylated or sialylated carbohydrates, which allows them to mediate relatively weak binding between endothelium and migrating cells, equipped with specific polysaccharide residues.[30] Lymphocyte homing or neutrophil extravasation are typical processes depending on transient selectin-mediated attachment.[28]

The structure and function of CD44 is extensively reviewed in this thesis.[31]

The CAMs play an important role in neoplasia, since intercellular adhesion and interactions of malignant cells with the surrounding extracellular matrix are crucial for invasion and metastasis. Spreading neoplastic cells have to reduce their cohesion with the primary tumor and crawl into the extracellular matrix, actively recognizing its components at the same time. On the other hand, in the bloodstream or within lymph, the more cohesive clumps of cancer cells have a greater chance of successfully anchoring themselves within capillary vessels. Tumor cells migrating within the vasculature mimic leukocytes in their adhesion to the endothelial surfaces, increasing the probability of a secondary focus

formation. Thus the spread of a neoplasm requires a delicately orchestrated balance between adhesion and antiadhesion. It is easy to imagine that the differences between behavior of a malignant cell and its normal counterpart should be mirrored by differences in quality and quantity of their adhesion molecules.

To date many proteins implicated in the biology of colon cancer have been proven to take part in adhesion processes. For example, carcinoembryonic antigen (CEA), strongly and almost constantly overexpressed by colon cancer cells, has been identified as a mediator of homotypic/ homophilic cell-to-cell interactions.[29-32] Conversely, an intercellular adhesion molecule E-cadherin as well as a family of cooperating intracytoplasmic proteins, the catenins, are often downregulated in colonic adenomas and carcinomas, providing a molecular basis for the increased mobility of neoplastic cells (reviewed in [33]). APC protein, which is a product of a tumor suppressor gene APC (adenomatous polyposis coli) and is crucial in the initial steps of colorectal carcinogenesis, associates with beta-catenin, itself complexed with the cell adhesion protein E-cadherin.[34] A strong candidate for another tumor suppressor gene, DCC (deleted in colorectal cancer), shows structural similarities to NCAMs (neural cell adhesion molecules) and likely performs analogous, adhesion-modifying functions.[35] Abnormal expression of integrins, the ubiquitous cell-to-matrix adhesion proteins, occurs in colon cancer cells and contributes to colon cancer progression.[36,37] One of the final steps in the metastatic process, adhesion to endothelial cells, is mediated by E-selectin. Notably, expression of E-selectin ligands by colon cancer cell lines has been positively correlated with their metastatic potential.[38]

Plan of the present thesis.

The present dissertation is focused on the role of CD44 in biology of colorectal cancer. CD44 is the main receptor for a ubiquitous extracellular polysaccharide, hyaluronan. It is a transmembrane glycoprotein, encoded by a single gene undergoing complex alternative splicing. At least 10 flanking exons are constantly expressed, whereas the middle portion of an extracellular domain can be composed of products of various

combinations of at least 10 alternatively spliced exons. The numerous isoforms of CD44 differ in their ability to bind hyaluronan, in their capacity to trap growth factors and its propensity to be shed from the cell membrane. Thus adhesion to hyaluronan, and possibly to other ligands of CD44, can be precisely regulated, modifying the interactions of cells with the surrounding environment. The article "*CD44 and the adhesion of neoplastic cells*"[31] , constitutes the first part of the thesis and reviews the current state of knowledge on CD44, dealing especially with its role in cancer.

The second part of the dissertation is devoted to verification of a hypothesis, assuming an involvement of CD44 overexpression in a murine model of colorectal cancer, induced by 1,2-dimethylhydrazine (*Changes in CD44 expression during carcinogenesis of the mouse colon* by Z. Rudzki, L. LeDuy and S. Jothy, 1997, submitted for publication). According to this premise, the neoplastic transformation of the murine colonic epithelial cell should be associated with the increase of CD44 mRNA and protein product. The 1,2-dimethylhydrazine induced neoplasia, which has morphological similarities to the human adenoma - carcinoma sequence, provides a unique opportunity for studying the molecular aspects of colorectal carcinogenesis at almost all stages, from the first dysplastic crypts to advanced, invasive carcinomas. If the animal model under study resembles human colorectal adenocarcinoma, this overexpression should encompass not only the standard form of CD44 but also its isoforms resulting from alternative splicing of mRNA. Furthermore, it is hypothesized, that in the murine model, the overexpression of CD44 takes place at the defined step of the dysplasia - adenoma - carcinoma sequence.

In order to verify the hypothesis, CD44 glycoprotein was examined using immunohistochemistry and the issue of transcription/ splicing pathology was addressed by RT-PCR followed by Southern blot. The findings in the murine model were compared to the available data on CD44 role in human colon cancer. To my knowledge this is the first report on CD44 expression in a chemically induced animal neoplasm.

References

1 Johnson RD, Geisinger KR. Colorectal adenocarcinoma: staging and histopathology. *Semin Roentgenol* 1996;**31**:94-102.

2 McMichael AJ, Giles GG. Colorectal cancer. *Cancer Surv* 1994;**19-20**:77-98.

3 Burkitt DP. Possible relationships between bowel cancer and dietary habits. *Proc R Soc Med* 1971;**64**:964-5.

4 Furukawa K, Yamamoto I, Tanida N, Tsujiai T, Nishikawa M, Narisawa T, Shimoyama T. The effects of dietary fiber from Lagenaria scineraria (yugao-melon) on colonic carcinogenesis in mice. *Cancer* 1995;**75**:1508-15.

5 Abdelali H, Cassand P, Soussotte V, Daubeze M, Bouley C, Narbonne JF. Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr Cancer* 1995;**24**:121-32.

6 Cunningham C, Dunlop MG. Molecular genetic basis of colorectal cancer susceptibility. *Br J Surg* 1996;**83**:321-9.

7 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;**87**:159-70.

8 Su LK, Johnson KA, Smith KJ, Hill DE, Vogelstein B, Kinzler KW. Association between wild type and mutant APC gene products. *Cancer Res* 1993;**53**:2728-31.

9 Powell SM, Petersen GM, Krush AJ, Booker S, Jen J, Giardiello FM, et al. Molecular diagnosis of familial adenomatous polyposis. *N Engl J Med* 1993;**329**:1982-7.

10 Gardner EJ. Follow-up study of a family group exhibiting dominant inheritance for a syndrome including intestinal polyps, osteomas, fibromas and epidermal cysts. *Am J Hum Genet* 1962;**14**:376-390.

11 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;**61**:759-67.

12 Konishi M, Kikuchi-Yanoshita R, Tanaka K, Muraoka M, Onda A, Okumura Y, et al. Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology* 1996;**111**:307-17.

13 Lynch HT, Smyrk T. Hereditary nonpolyposis colorectal cancer (Lynch syndrome). An updated review. *Cancer* 1996;**78**:1149-67.

14 Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;**268**:1336-8.

15 Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997;**275**:967-969.

16 Burt RW. Familial risk and colon cancer. *Int J Cancer* 1996;**69**:44-6.

17 Hardcastle JD, Justin TA. Screening high-risk groups for colorectal neoplasia. *Am J Gastroenterol* 1996;**91**:850-2.

18 Online Mendelian Inheritance in Man, OMIM (TM). Center for Medical Genetics, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 1996. World Wide Web URL: http://www3.ncbi.nlm.nih.gov/omim/

19 Harpaz N, Talbot IC. Colorectal cancer in idiopathic inflammatory bowel disease. *Semin Diagn Pathol* 1996;**13**:339-357.

20 Lewin KJ, Riddel RH, Weinstein WM: Gastrointestinal Pathology and Its Clinical Implications. New York, Igaku-Shoin Medical Publishers, 1992.

21 Fenoglio-Preiser CM, Pascal RR, Perzin KH: Tumors of the Intestines. Washington, Armed Forces Institute of Pathology, 1990.

22 Stachura J, Gedliczka O, Bogdal J, Tarnawski A. Wtorna prewencja raka jelita grubego. *Pat Pol* 1992;**43**:65-71.

23 Levine DS, Haggitt RC: Colon. In: Histology for Pathologists. Edited by SS Sternberg. New York, Raven Press, 1992, pp 573-592.

24 Johnston PG, Allegra CJ. Colorectal cancer biology: clinical implications. *Semin Oncol* 1995;**22**:418-32.

25 Pignatelli M, Vessey CJ. Adhesion molecules: novel molecular tools in tumor pathology. *Hum Pathol* 1994;**25**:849-56.

26 Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu Rev Biochem* 1990;**59**:237-52.

27 Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992;**69**:11-25.

28 Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD: Molecular Biology of the Cell. New York, Garland Publishing, 1994, pp 950-999.

29 Benchimol S, Fuks A, Jothy S, Beauchemin N, Shirota K, Stanners CP. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 1989;**57**:327-34.

30 Bevilacqua M, Butcher E, Furie B, Furie B, Gallatin M, Gimbrone M, et al. Selectins: a family of adhesion receptors. *Cell* 1991;**67**:233.

31 Rudzki Z, Jothy S. CD44 and the adhesion of neoplastic cells. *J Clin Pathol: Mol Pathol* 1997 (in press).

32 Jothy S, Yuan SY, Shirota K. Transcription of carcinoembryonic antigen in normal colon and colon carcinoma. In situ hybridization study and implication for a new in vivo functional model. *Am J Pathol* 1993;**143**:250-7.

33 Jothy S, Munro SB, LeDuy L, McClure D, Blaschuk OW. Adhesion or anti-adhesion in cancer: what matters more ?. *Cancer Metastasis Rev* 1995;**14**:363-76.

34 Hulsken J, Birchmeier W, Behrens J. E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* 1994;**127**:2061-9.

35 Fearon ER, Pierceall WE. The deleted in colorectal cancer (DCC) gene: a candidate tumour suppressor gene encoding a cell surface protein with similarity to neural cell adhesion molecules. *Cancer Surv* 1995;**24**:3-17.

36 Fujita S, Watanabe M, Kubota T, Teramoto T, Kitajima M. Alteration of expression in integrin beta 1-subunit correlates with invasion and metastasis in colorectal cancer. *Cancer Lett* 1995;**91**:145-9.

37 Nigam AK, Savage FJ, Boulos PB, Stamp GW, Liu D, Pignatelli M. Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer. *Br J Cancer* 1993;**68**:507-14.

38 Sawada R, Tsuboi S, Fukuda M. Differential E-selectin-dependent adhesion efficiency in sublines of a human colon cancer exhibiting distinct metastatic potentials. *J Biol Chem* 1994;**269**:1425-31.

CD44 and the adhesion of neoplastic cells.

by Zbigniew Rudzki and Serge Jothy

Department of Pathology, McGill University, Montreal, Quebec, Canada

running title: *CD44 and cancer*

Supported by grants form the Medical Research Council and the National Cancer Institute of Canada. Z.R. is supported by a Hans and Eugenia Jütting Fellowship.

corresponding author: Serge Jothy, M.D., Ph.D., Professor of Pathology, Department of Pathology, McGill University, 3775 University Street, Montreal, Quebec, Canada H3A 2B4.

key words: CD44, cell adhesion, cancer

Abstract

CD44 family of transmembrane glycoproteins acts as receptors for hyaluronan. CD44 can also bind some other extracellular matrix ligands (chondroitin sulfate, heparan sulfate, fibronectin, serglycin, osteopontin) with lower affinity. CD44 is encoded by a single gene containing 20 exons, 10 of which (v1-v10) are variant exons inserted by alternative splicing. The standard, ubiquitously expressed isoform of CD44, does not contain sequences encoded by these variant exons. Numerous variant isoforms of CD44 containing different combinations of exons v1 to v10 inserted into the extracellular domain can be expressed in proliferating epithelial cells and activated lymphocytes. CD44 plays a significant role in lymphocyte homing. Both alternative splicing and glycosylation influence receptor function of CD44, usually reducing its affinity to hyaluronan. The cytoplasmic domain of CD44 communicates with the cytoskeleton via ankyrin and proteins belonging to ezrin-moesin-radixin family. Relatively little is known about the intracellular events following interactions of CD44 with its ligands.

Some variant isoforms, especially those containing sequences encoded by v6-v10, are overexpressed in both human and animal neoplasms. In a rat pancreatic adenocarcinoma model one of the variant CD44 isoforms was proved to be determinant in the metastatic process. For some human neoplasms (carcinomas of digestive tract, non-Hodgkin's lymphomas, thyroid carcinomas and others) correlations have been made between particular patterns of CD44 variants produced by neoplastic cells and clinico-pathological parameters of tumors, such as grade, stage, presence of metastases and survival. *In vitro* studies indicate that modifications of CD44 expression result in different ligand recognition and influence cell motility, invasive properties and metastatic potential of experimental tumors. Investigation of CD44 neoexpression can be useful both in early cancer diagnosis and in predicting tumor behavior. It can also contribute to better understanding of molecular mechanisms leading to neoplastic transformation.

key words: CD44, cell adhesion, cancer

One of the important basic features of tumor cell biology is the property of malignant cells to change their adhesion to other cells and to the extracellular matrix. This property gives to cancer cells the potential to detach from their original site of tumor growth and to invade surrounding tissues. CD44 is a typical example of a cell adhesion protein which undergoes dramatic structural and functional changes during malignant transformation.

Terminology.

CD44 is a family of glycoprotein molecules, which received its name during the Third International Workshop on Leukocyte Differentiation Antigens.[1] This nondescriptive term has replaced previously used names, including: lymphocyte homing receptor ($gp90^{Hermes}$), phagocytic glycoprotein (Pgp-1), extracellular matrix receptor III (ECMRIII), hyaluronate receptor (H-CAM) and HUTCH-1. CD44 was first described in the early 1980s, based on the reactivity of an antibody, called F 10.44.2 [2], (reviewed in: [3,4]). As those separately described and named molecules with diverse functions were found to be the product of one gene, the interest in CD44 rapidly increased, resulting in over 800 publications during the last 5 years.

Overview of CD44 genomic organization and protein structure.

CD44 is a product of a single gene, located on the short arm of human chromosome 11.[5] Genomic DNA studies have documented that CD44 is encoded by at least 20 exons. The first five (s1-s5), as well as exons 16-20 (s6-s10) are almost invariably expressed by a large number of non-epithelial, including hematopoietic, cells; their product is referred to as the "standard" or "hematopoietic" form of CD44 (abbreviated CD44s or CD44H).[6] Exons 6-15, more often called v1-v10, are alternatively spliced.[7] Despite their name, some "standard" exons can occasionally participate in alternative splicing, and some of the variant ones contain "cryptic" splice sites within them.[6] The structure of CD44 is illustrated in figure 1.

Unlike the mouse CD44 gene, the human exon v1 contains a stop codon at the 17th amino acid residue and probably does not give rise to a protein product.[8] There is no evidence that human CD44 isoforms contain the v1 sequence. Since human genomic v1 was discovered only after the finding of the homologous murine exon (op. cit.), it is sometimes designated as exon 5a or 6a, and subsequent exons receive the numbers from 6 to 19 or from 7 to 20, which can lead to some terminological confusion.[9] Similarly, there is no widely accepted terminology for the large number of resulting isoforms described, the only exception being the isoform containing the product of exons v8-v10, known as an "epithelial" variant (CD44E). However, even this isoform (CD44v48-10) has been called "CD44R1" by another group.[10] The largest human isoform, containing the insert encoded by all alternatively spliced exons from v2 to v10 was called "epican".[11] The number of isoforms documented so far approaches 100, although theoretically there could be easily more than 1000 combinations.

The standard form of human CD44 is composed of 361 aminoacids (37-42 kDa).[6] In the human the largest insert, resulting from alternative splicing, contains an additional 381 aminoacids. The CD44 molecule can be subdivided into 4 distinct regions.[12] The N-terminal found in the extracellular domain is encoded by exons s1 to s5 and is highly conserved among the species.[13] Three pairs of cysteine residues, forming disulfide bonds shape the CD44 ectodomain into globular loops. Two chondroitin sulfate chains can be attached to the product of exon s5. The N-terminal region also shares a limited (~30%) sequence homology with the B-loop of the cartilage link protein and other extracellular hyaladherins, like aggrecan and versican.

The N-terminal region of CD44 contains two binding sites for hyaluronic acid, also called hyaluronan, within regions corresponding to exons s2 and s5. These hyaluronan binding motifs, called "B(X7)B", consist of seven aminoacids, none of them acidic, flanked by two basic aminoacids--either arginine or lysine.[14] One of them, located closer to the N-terminus, lies within the region homologous with the cartilage link protein and contains arginine at position 41, which is necessary for hyaluronan recognition.[15] The second more

proximal region located in a domain with no significant homology with other hyaladherins seems to play an accessory role (op. cit.). Recently a third hyaluronan binding B(X7)B motif was described, partially overlapping that previously identified within exon s5 product.[16] Similar hyaluronan recognition sequences have also been identified in another cell surface hyaluronan receptor--RHAMM ("Receptor for hyaluronan - mediated motility").[14]

The membrane proximal domain, encoded by exons s6 and s7, is less well conserved among the species (~50%) and contains the site for insertion of the alternative splicing products, between s5 and s6. Up to 381 aminoacids, encoded by various combinations of alternatively spliced exons v1 to v10 can be added at aminoacid 223. These alternatively spliced exons do not show any significant homology with any other protein sequences and their function remains still largely unknown. The limited similarity between the alternatively spliced exons themselves suggests that they might have evolved from a common ancestor sequence by means of duplication.[8] At least three of them have potential binding sites for heparan sulfate and chondroitin sulphate through the SGXG motif found within the product of exon v3.[17] Fucose rich H-blood group antigens can also bind to v6 and v10 contains two potential binding sites for chondroitin sulfate.[18] The proximal extracellular region, encoded by standard exons is also a target of post-translational modifications, binding up to three chains of chondroitin-4-sulfate.

The short hydrophobic transmembrane domain composed of 23 aminoacids is encoded by exon s8, the remaining 3 aminoacids of this exon coding for the beginning of the cytoplasmic tail.[6] Exons s9 and s10, despite their names, can also be alternatively spliced, resulting in the short-tail (additional three aminoacids, exon s9) or much more prevalent, long-tail (additional sixty-seven aminoacids, exon s10) version of the cytoplasmic domain.[19] It is unclear, whether the short-tail form of CD44 is expressed on the cell surface, and if it has any functional role (op. cit.). Four serine residues in the cytoplasmic domain are potential substrates for various kinases and there is a limited homology between this part of CD44 and sequences found in the G-protein family.[20] The sequence between

aminoacids 305 and 355 of the CD44 cytoplasmic domain also contains two putative ankyrin binding sites.[21]

The molecular mass of the mature protein usually exceeds 85 kDa, as numerous potential N- and O-glycosylation sites of the extracellular domains are substituted by carbohydrates. Potential O-glycosylation sites, distinguished by high Ser/Thr content are especially abundant within the products of alternatively spliced exons, v10 containing the largest number of Ser/Thr rich regions.[22] The chondroitin- and heparan-sulfate chains contribute to the final molecular mass of some CD44 species, the former increasing it approximately by an additional 110-120 kDa.[23] Thus the most prevalent form of CD44 (CD44s) is typically encountered as the 85-95 kDa "backbone" or as a 190 kDa variant, both carrying the chondroitin sulfate chains. The suggestion [24] that the heavier variant represents the disulfide linked homodimer of the former has not been confirmed so far.

The CD44 protein has been described in human, mouse, rat, hamster, goat, pig, dog, sheep, cattle and horse, with the N-terminal and cytoplasmic domains showing the closest sequence homology (80-90%) among species.[4] Also the variant region (v1-v10) is similarly well conserved, when mouse and rat sequences are compared. The human-rodent homology of this region is approximately 65%.[8] Interestingly, most of the O-glycosylation sites, as well as the substitution sites for chondroitin sulfate chains belong to the well conserved regions, whereas this is not always the case for the targets for N-glycosylation (op. cit.). A strikingly high interspecies homology characterizes the intronic sequences, especially the intron v4-v5 (68% identity between human and mouse). Since the introns are usually poorly conserved their evolutionary preservation in the CD44 variant region suggests the possible role of intron-encoded information in splicing regulation (op. cit.).

So far no CD44 or CD44-like protein has been described in organisms other than mammals.

Expression of CD44 in various tissues.

Taken as a family of molecules, CD44 is a ubiquitous cell protein. Recently, a panel of monoclonal antibodies directed against the standard isoform of CD44, as well as the products of variant exons was used to examine both adult and embryonal human tissues.[25,26] Generally, most epithelial and nonepithelial tissues express CD44s, whereas positive reactions with antibodies against exon v6, v9 and v4 are far more restricted. The following adult tissues have no detectable expression of standard or variant CD44: ependyma, epithelium of the choroid plexus, surface epithelium of stomach and intestine, hepatocytes, endocrine portion of pancreas, pancreatic acinar cells, adrenal glands, kidney proximal tubules and collecting ducts as well as Bowman's capsule, ovarian germinal epithelium, testicular germ cells, striated muscle (both cardiac and skeletal), Sertoli cells and astrocytes. The standard form of CD44 is probably the only one encountered in nonepithelial tissues, with the singular exception constituted by microglia showing weak reaction with anti v6 antibodies.[25]

Epithelial tissues differ markedly among themselves as to the expression of variant isoforms, but generally the products of exon v9 parallel the occurrence of CD44s. In normal breast, the "epithelial" form of CD44 (CD44E) seems to be even more abundant than the standard one, at least at the mRNA level.[27] Breast duct myoepithelium differs from the epithelial layer by the display of a v6 containing CD44 isoforms.[25] Besides, v6-containing isoforms are more restricted and are found mainly in squamous and transitional epithelia and ductal parts of glands, the pancreas being a typical example.[28,29] Oesophageal epithelium, basal and lower intermediate urothelium as well as the lower layers of epidermis belong to the very rare localizations of v4-containing isoforms.[25,30] One of them is epican (v2-v10), expressed in the epidermis from the basal to the granular cell layer.[11] Generally, in epithelial tissues the variant isoforms tend to be more strongly expressed in proliferating cells.[26] CD44 expression in fetal tissues is usually similar but less prominent than in the adult counterparts. A notable exception is embryonal skeletal muscle which is strongly positive for CD44s.[25]

Isoforms containing v1-encoded sequences have not been identified in human tissue, probably because human v1 contains the stop codon.[8] In mice this exon is not interrupted by the stop signal. However, expression of murine isoforms containing v1 is highly restricted and was found only in the stomach.

In polarized epithelial cells, CD44 occupies mainly the lateral plasma membranes. The cytoplasmic domain is responsible for this restricted localization, since mutants lacking the C-terminal tail are distributed instead on the apical part of the cells.[31] In fibroblasts, CD44 preferentially occupies the cytoplasmic projections and this localization requires the integrity of its intracellular domain.[32]

It is worthwhile emphasizing that almost all cells of connective tissue, such as fibroblasts, endothelial cells and macrophages contain large amounts of CD44s. Hence there is no CD44-free organ or cellular microenvironment in the human body. Hemopoietic cells are characterized by a high expression of CD44; their membrane contains 10^4-10^5 molecules, mainly of the standard type.[33] It is relevant to notice that more than one CD44 isoform can be expressed by a single cell.[34]

Soluble CD44 (5 μg/ml) can be detected in human serum.[35] It is probably released from the cell surfaces by proteolytic enzymes and its biological significance as well as the exact cell of origin is still unknown. Interestingly, in patients with gastric and colon carcinoma the serum concentration behaves as a marker of a neoplastic process, as values are increased nearly 10 fold, and correlate positively with tumor burden.[36]

Physiologic functions of CD44.

CD44 as a hyaluronan-binding glycoprotein.

Hyaluronan is the main ligand of CD44. It is an abundant component of the extracellular matrix and consists of a linear polymer of repeated disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine. It differs from other glycosaminoglycans by its lack of covalently linked peptide chains. A single extended molecule can reach a length of 10 μm and a mass of up to 10 million daltons. A hydrated

hyaluronan molecule can form a random coil of up to 500 nm in diameter. Aggregates composed of hyaluronan attached to proteoglycans by the 40 kDa link protein perform important mechanical functions in various tissues contributing to the resistance of soft tissues. They also act as an osmotic buffer and a filter regulating the diffusion of macromolecules.

Hyaluronan-binding properties of what was called "fibroblast surface protein", later found to be identical to CD44, were first reported in the 1980's.[37,38,39] CD44 mediates the adhesion of stromal cells to the lymphoid precursors in bone marrow.[40] This interaction is blocked by both hyaluronidase and anti-CD44 antibodies, and is optimal at neutral pH. Some anti-CD44 antibodies potentiate ligand recognition, as illustrated by the IRAWB14 monoclonal antibody, which recognizes an epitope situated between hyaluronan binding motifs.[41]

Most binding assays have been performed in the presence of divalent cations. However, CD44 mediated adhesion can occur in the absence of calcium, with both of its known ligands: hyaluronan and osteopontin.[40,42,43,44] The binding affinity is relatively weak when compared with the forces resulting from mechanisms involving cadherins or integrins. CD44-hyaluronan interactions are approximately five times stronger then those mediated by RHAMM, the second widespread hyaluronan receptor.[9,45]

The B(X7)B motifs, located on the CD44 extracellular domain are essential, but not sufficient for optimal hyaluronan binding. Other factors included are which CD44 isoform is involved, its posttranslational modifications and cellular context. The standard form of CD44 binds hyaluronan, whereas the ligand affinity of some variant isoforms, especially the "epithelial" one (CD44E) is much lower.[24,46] It is presently unclear, why the insertion of the additional amino acids encoded by variant exons in a location remote from the hyaluronan-binding sites affects CD44-hyaluronan interactions. An important issue is to define the role played by variant CD44 isoforms in the attachment to hyaluronan. Studies addressing this issue have led to different conclusions. For instance the murine homologue of human CD44E and other heavier murine isoforms bind to the ligand, when transfected into the

AKR1 lymphoma cells.[47] Also the CD44R1 isoform, when expressed in COS7 (SV40-transformed simian fibroblastoid cell line) or TIL1 (murine lymphoma) cells can bind hyaluronan.[48,49] The CD44R1 isoform was regarded as similar, but not identical to CD44E. Recently it was acknowledged that the aminoacid composition of these two CD44 variants is the same (Aruffo A; personal communication). Whatever variant exon is spliced in CD44 transcripts, post-transcriptional modifications can still account for further changes in hyaluronan binding.

Various sugar moieties carried by CD44 modify its capacity to bind hyaluronan, probably by altering the folding or charge of the receptor molecule. O-glycosylation, and to a lesser extent N-glycosylation of the standard form of CD44 are necessary for transfected murine T lymphoma cells to bind to hyaluronan.[50] Interestingly, glycosylation of some of the variant domains of CD44 has the opposite effect.[22] Thus variable glycosylation of the CD44 molecule, particularly its "epithelial" variant, can serve as a mechanism regulating lectin function of this glycoprotein parallel to alternative splicing.

Similarly, the attachment of chondroitin sulfate or heparan sulfate, both negatively charged molecules, to the v3-encoded region of CD 44 can have an adverse effect on hyaluronan binding.[17]

Binding of CD44 positive cells to hyaluronan and their tumorigenic potential also depend on whether CD44 remains attached to the plasma membrane or is shed in the extracellular space.[51] Variant CD44 isoforms containing sequences encoded by v7 to v10 shed quite easily into the extracellular space, probably as a result of proteolytic cleavage of CD44 at the junction between the transmembrane and cytoplasmic domains. The released fragments block CD44-dependent tumor formation, which relies on adhesion of neoplastic cells to hyaluronan. In contrast, the standard CD44 isoform does not easily shed, promoting local tumor growth upon subcutaneous injection into nude mice. Interestingly, cells transfected with isoforms containing products of v3, which completely fail to bind hyaluronan and do not release CD44, are the most efficient in forming bone marrow metastases after intravenous injection. This leads to the suggestion that the extensive

glycosylation of the v3-coded region has several corollaries: abrogation of hyaluronan binding, specific protection from proteolysis and promotion of unique metastatic properties, possibly by trapping growth factors.[51]

The cytoplasmic domain is essential for binding to soluble or immobilized hyaluronan and for hyaluronan/CD44-mediated motility. This process involves clustering of CD44 molecules at the surface of the cell.[52]

As expected, alterations in hyaluronan binding also relate to changes in the number of CD44 molecules expressed on the cell membrane. Activation of lymphocytes by phorbol esters increases the expression of the CD44 standard isoform and results in increased binding to hyaluronan.[53]

Finally the affinity of CD44 for hyaluronan depends on the size of the ligand molecule. Hyaluronan has no constant molecular mass *in vivo*, and generally, the longer the polymer chain, the stronger is CD44 affinity to it. The minimal hyaluronan chain, still recognized by CD44 is 6 sugar residues in length.[9]

Binding to hyaluronan is unlikely to be the only functional consequence of CD44 expression. It is conceivable that associated events are related to it and include hyaluronan degradation, cell motility and intracellular signalling.

Hyaluronan is degraded mostly in lymph nodes and to a lesser extent in the liver, spleen, kidneys and lungs.[9, 54] Transformed cells are also efficient in hyaluronan degradation. A series of breast cancer cell lines was shown to degrade hyaluronan into short oligosaccharide chains, and Hermes-1 antibody which recognizes the N-terminal region of the CD44 ectodomain, was shown to block this process efficiently.[55] Moreover, in this study the positive correlation between the CD44-associated hyaluronan degradation and the invasive potential of the cell lines was demonstrated.

Hyaluronan promotes cell locomotion and its accumulation accompanies processes such as wound healing, embryogenesis (neural crest cell migration and migration of mesenchymal cells forming the cardiac septum), and angiogenesis (reviewed in [56]). However, a determinant role for CD44 in these processes remains speculative. In the

mouse embryo CD44 (both the standard form as well as the heavier isoforms) colocalizes with hyaluronan during the various stages of development.[57] The only strong evidence supporting CD44-dependent cell migration promoted by hyaluronan comes from studies using neoplastic cells.[46]

With respect to intracellular signalling, experiments with murine T lymphoma cells stimulated by hyaluronan show a rapid increase in intracytoplasmic calcium, which is accompanied by CD44 capping on the cell surface and increased binding of the cells to immobilized hyaluronan. Since this response can be blocked with anti-CD44 antibodies, it is very probable that CD44 can also serve as an "outside-in" signal transducer.[58]

Interactions of CD44 with other ligands.

So far hyaluronan remains the only component to which CD44 binds specifically. Cell binding to collagen type I, laminin and fibronectin are also CD44-dependent, but are weaker and rely on the presence of linked chondroitin sulfate chains.[59] These interactions can also be mediated by CD44 isoforms containing products of variant exons. Some colon carcinoma cell lines expressing CD44E show binding to type IV collagen and laminin that can be blocked by anti-CD44 antibodies.[60] Also the CD44-negative murine lymphoma cells TIL1 transfected with CD44R1 (an isoform containing the product of exon v10 with the chondroitin sulfate chain attachment site) undergo spontaneous homotypic aggregation, probably mediated by the mutual recognition of CD44R1 molecules and chondroitin sulfate chains.[49]

Recently serglycin, the sulfated proteoglycan of leukocyte secretory granules, was identified as another ligand for CD44. Serglycin can be exocytosed and interacts with CD44 on cell membrane leading to activation of lymphocytes. Here again the binding to CD44 is dependent on the chondroitin sulfate chains carried by the serglycin molecule.[61]

Heparan sulfate, attached to the region encoded by exon v3, enables CD44 heavy isoforms to bind growth factors, possibly presenting them at sites of inflammatory

reactions. Heparin-binding epithelial growth factor (HB-EGF) and basic fibroblast growth factor (b-FGF), which are determinants of angiogenesis, belong to those so far identified.[62]

According to a recent report, osteopontin which is an ubiquitous extracellular matrix glycoprotein may be another ligand for CD44.[44] Osteopontin was originally described as a protein involved in bone apatite crystal formation, but is also produced by a number of epithelial cells, vascular smooth muscle cells and lymphocytes. Osteopontin is involved in such diverse processes as suppression of nitric oxide synthesis and promotion of cancer cell migration (reviewed in [63]). In addition osteopontin is a ligand for integrins and participates in calcium-dependent adhesion.[64,65] By contrast, CD44-mediated adhesion does not require divalent cations nor does it involve a GRGDS sequence. Also the presence of intact chondroitin sulfate chains attached to osteopontin is not necessary. However, examination of human osteopontin aminoacid composition [66] shows that it contains at least two potential "B(X7)B" hyaluronan binding motifs. Methodology used by Weber et al. does not rule out the possibility that CD44-osteopontin binding is indirect and mediated by hyaluronan chains that can bridge osteopontin molecules with CD44. The thrombin cleavage site present in osteopontin is confined to one of those motifs. Since osteopontin produced by tumor cells and peritumoral macrophages promotes neoplastic cell motility when cleaved by thrombin [67], the issue of CD44-hyaluronan-osteopontin interactions at the advancing tumor margin seems to be an interesting field for future research.

CD44-mediated lymphocyte homing.

Lymphocyte homing refers to a process in which migrating lymphoid cells recognize a specific ligand on the surface of high endothelial venules and extravasate to the Peyer's patches and lymph nodes in some organs.[68] Lymphocytes and their precursors, like other cells of hematopoietic origin, express CD44 from the very early to late stages of their maturation. The level of CD44 expression on T lymphocytes depends also on their activation. Antigenic stimulation or interleukin 2 combined with anti-CD3 antibodies not only increase the overall amount of CD44 on the lymphocyte membrane, but also induces

variant isoforms.[69,70] This reaction is maximal 24 hours after stimulation and is followed by blast transformation and DNA synthesis. Stimulated T cells expressing more CD44 adhere more avidly to human endothelium.[71] Both responses can be blocked by Hermes-3 antibody which recognizes the membrane proximal CD44 domain. This antibody does not interfere with CD44-hyaluronan interactions. Earlier, the so called "Hermes antigen", later identified as CD44 has been shown to be involved in the homing of peripheral blood lymphocytes.

Non-inflammatory lymphocyte recirculation probably does not depend exclusively on CD44, since CD44-negative lymphocytes isolated from anti-CD44 treated normal mice still show the ability to home to the lymphoid organs. However, during inflammatory reactions the extravasation of leukocytes lacking CD44 is impaired and they are incapable of generating a cutaneous delayed-type hypersensitivity response in non-lymphoid tissues.

CD44 during lymphopoiesis and hematopoiesis.

The earliest population of thymocytes expresses CD44s. Anti-CD44 antibodies are effective in preventing the repopulation of thymus by T-cell precursors in mice.[72] During the subsequent maturation, naive T-cells lose their CD44, which is then regained by memory T-lymphocytes. The latter cells express both standard and variant exon containing isoforms, whereas in the early precursor thymocytes, the v9 product can be found only on the thymic medullary cells.[25,73]

Similarly, anti-CD44 antibodies can interfere with B cell development and mature B cells can transiently upregulate CD44 variant isoforms, including those containing the sequence encoded by exon v6.[69,74]

Cells from the myeloid and erythroid lineages also show a high expression of CD44. The specific role played by CD44 relates to adhesive interactions between hematopoietic and stromal cells in the bone marrow.[74]

Associations between CD44 and the cytoskeleton.

Cell motility experiments have shown that CD44 is connected to the cytoskeleton (reviewed in [32]). This connection is not "stiff" and differs in various regions of a moving cell: CD44-cytoskeleton association is stronger at the leading edge of the cell membrane as opposed to the trailing one. The exact nature of interactions between CD44 and cytoskeleton proteins awaits further clarification. There is evidence that CD44 binds to ankyrin, which connects the submembranous part of the cell scaffolding with the actin filament meshwork.[75,76] The ankyrin-binding domain of the intracytoplasmic portion of the CD44 molecule contains at least two regions recognizing the ligand with different affinity. Interestingly, the presence of the ankyrin-binding domain is necessary for hyaluronan binding suggesting that the intra- and extracellular functions of CD44 are functionally connected.[21] The ability to bind hyaluronan requires that at least 14 amino acids of the intracytoplasmic domain be preserved or that CD44 molecules be dimerized via the disulfide bonds.[77]

By contrast, treatment of activated lymphoid cells with cytochalasin B does not affect their ability to bind hyaluronan, suggesting that the interaction of CD44 with actin filaments is not indispensable for recognition of this ligand.[78]

New information on the association of CD44 with cytoskeletal proteins was provided when experiments showed that CD44 colocalizes with the members of ERM (ezrin-radixin-moesin) family in hamster and mouse cells.[79] These proteins are involved in plasma membrane-actin connections such as formation of microvillous projections, adherens junctions, ruffling membranes and cleavage furrows.[80] Both standard CD44 as well as v9/v10 containing isoforms associate with ERM proteins, the affinity of the heavier isoforms being substantially greater.[79] It is still unclear as to what is the function of the postulated CD44-ERM complex and whether ankyrin participates in its formation.

CD44 involvement in neoplastic invasion and metastasis.

The original description of CD44 as a metastasis molecule.

The participation of CD44 in tumor progression and metastasis was first documented in an animal tumor model. This was established during a search for antigenic determinants discriminating between metastasizing and non-metastasizing subclones of a rat adenocarcinoma cell line.[81] The generation of cDNA clones identified the metastasizing cell specific protein as a variant of CD44 containing the product of exon v6. Further experiments confirmed that expression of this particular CD44 variant molecule in transfected non-metastasizing cells confers the ability to form distant metastases. Furthermore, non-cytotoxic antibodies specific for this CD44 variant inhibited formation of secondary neoplastic foci when injected together with tumor cells.[82] Thus, in this particular system, a single molecule, CD44 carrying variant exon v6, proved to be causal for the formation of distant metastases. Applying this information to the human species has to be subjected to caution. Although human pancreatic adenocarcinomas show almost consistently v6 immunopositivity, expression of v6 containing isoforms can also be detected in normal human ductal pancreatic cells.[28]

One of the first indications that aberrant splicing of CD44 is relevant to human tumor diagnosis was reported in 1992.[83] RT-PCR analysis followed by Southern blotting demonstrated that human colon and breast carcinomas are usually associated with the generation of a number of large CD44 splicing isoforms.

These initial observations were followed by CD44 investigations of non-epithelial neoplasms. As discussed below the results obtained with different types of cancer have helped to understand that more than one CD44 isoform is involved in tumor progression; tumor spreading related to expression of CD44 is mediated by various CD44 isoforms, depending on the type of the neoplasm.

Lymphomas.

The numerous attempts that have been made for a consensus classification of non-Hodgkin lymphomas reflect the frequent unpredictability in behavior and insufficient correlation between morphological and clinical features of these neoplasms. Consequently, active searches are being pursued to identify new markers enabling more accurate lymphoma classification and prognostic stratification. The role of CD44 in lymphocyte homing together with the transient upregulation of large CD44 isoforms in activated lymphoid cells provided a rationale for assessing its potential participation in lymphoma progression. Basic science experiments gave a rationale to use CD44 as a prognostic marker. When the CD44 negative Namalwa lymphoma cell line is transfected with standard CD44, its metastatic potential is greatly enhanced.[84]

Nodular lymphomas, which are relatively indolent, are typically CD44 negative in contrast to the usually more aggressive diffuse lymphomas which are strongly CD44 positive.[85] Overall CD44 expression correlates with poor prognosis in gastrointestinal lymphoma.[86] A substantial fraction of gastrointestinal lymphomas reported in this study[86] (52%) showed no expression of CD44 and were characterized by a more favorable clinical outcome.

More detailed data can be obtained using reagents that are specific for alternatively spliced exons present in CD44 variant isoforms. The CD44 transcripts found in diffuse large cell lymphomas contain alternative splicing products which are not expressed in normal resting lymphocytes and resemble those found in activated B cells.[87] Overexpression of isoforms containing protein product of exons v3, v6 and v9 (but not v4) was observed in high grade lymphomas, as opposed to the low grade lymphoid neoplasms.[70] [88] Additionally, multivariate analysis of morphological, clinical and molecular parameters obtained in a large cohort of patients with high grade non-Hodgkin's lymphoma demonstrates that the presence of CD44-v6 product is an independent prognostic factor.[89] Interestingly, CD44-negative human Burkitt's lymphoma cells transfected with large variant CD44 isoforms show weaker adhesion to hyaluronate and increased aggressiveness, as

documented by *in vivo* tests.[51] The causal relationship between these two phenomena has not been proven but it is tempting to speculate that the decreased affinity to extracellular matrix can facilitate mobilization of lymphoma cells and promote their spread via blood and lymphatic vasculature.

Overall these human studies support the experimental observations and the notion that high expression of CD44 correlates with poor prognosis in lymphoma.

However, determinant factors others than CD44 relate to the aggressiveness of lymphoma at least in animal models. For instance, the MDAY-D2 murine lymphoma line in which CD44 is inactivated retains both its local invasiveness and metastatic potential, despite a complete loss of hyaluronan binding ability.[90]

The level of soluble standard form and v6 containing CD44 isoforms can be measured in the blood of lymphoma patients and behaves as a useful marker in monitoring the effects of therapy.[91]

In contrast to non-Hodgkin's lymphomas, the role of CD44 and its variants in the biology of Hodgkin's disease is not well known. The CD44 status of Reed-Sternberg cells is unclear, as is the relevance of the CD44 molecule to the prognosis of Hodgkin's disease.

Melanoma.

Human nonneoplastic melanocytes as well as some established melanoma cell lines are characterized by the presence of standard CD44 and the absence of variant isoforms.[34,92] In contrast, examination of various human melanocytic lesions from benign pigmented nevi to metastatic deposits of melanoma, demonstrates a complex pattern of CD44 expression. Isoforms resulting from alternative splicing and containing products of exon v5 can be detected in some nevi and their expression increases in overtly malignant lesions.[92] Conversely, v10 containing variant isoforms which are strongly upregulated in benign nevi, are less commonly expressed in atypical nevi, early melanomas, advanced melanomas and metastases. Products of exon v6 have not been detected in human melanocytic lesions but are identified in some melanoma cell lines, correlating positively with metastatic potential

after injection in nude mice. The same *in vivo* tests showed an even stronger relationship between v5 and metastatic phenotype. This is in keeping with the recent observation that CD44 v5 expression correlates with lymph node metastases in human melanomas, suggesting that CD44v5 may be responsible for binding of neoplastic melanocytes to antigen presenting cells within lymph nodes.[93] Binding could be followed by secretion of cytokines promoting survival of the metastatic focus.

The study of melanomas gives an opportunity to investigate how CD44 expression might relate to the presence of its ligand, hyaluronan, in the tumor microenvironment and how tumor cells themselves contribute to hyaluronan deposition. Highly metastatic melanoma cell lines produce more hyaluronan as opposed to cell lines showing poor or no metastatic capacity.[94]

There is a direct link between hyaluronan binding and the aggressiveness of human melanoma cell lines. Cells transfected with CD44 mutated at arginine 41 in exon s2, an essential site for hyaluronan recognition, not only completely lose lectin function but also have a dramatically reduced aggressiveness.[95] Markedly different behavior can be observed when human melanoma cells are sorted according to the intensity of CD44 expression. The clones characterized by the highest CD44 expression show, in addition to a stronger adhesion to hyaluronan, a more vigorous motility and homotypic aggregation. They also have an increased capacity to colonize lungs of nude mice injected subcutaneously with tumor cell suspensions.[96]

Colorectal carcinoma.

Colorectal carcinoma develops usually through a well recognized sequence of events: flat or slightly elevated foci of dysplastic crypt(s), adenoma with increasing grade of dysplasia, intramucosal carcinoma, and finally overtly malignant neoplasm invading the deep layers of the intestinal wall and spreading to the neighboring lymph nodes and distant organs. The prognosis of patients with intramucosal carcinoma is usually excellent and the prognosis of those with invasion beyond bowel wall and distant metastases is invariably

poor. By comparison, the behavior of the most frequent colorectal cancers which are diagnosed at an intermediate stage, remains often unpredictable.

Normal cells of adult human large intestinal epithelium express standard CD44 and less prominently v5, v7 and v9-containing isoforms in the basal region of epithelial crypts. Isoforms containing the products of exons v4 and v6 are not detected in adult colorectal epithelium by means of immunohistochemistry.[97 98] Fetal colonic epithelial cells are CD44 negative.[25 26]

Immunohistochemical examination of paraffin embedded colorectal tissue specimens, using a monoclonal antibody recognizing all isoforms of CD44 molecules, demonstrates that its accumulation can be found in early, small adenomas, becoming more frequent in larger lesions and carcinomas.[99] Furthermore CD44 overexpression precedes other genetic changes, like K-ras mutation, DCC deletion and expression of abnormally stable p53. It is worth emphasizing that among these, K-ras mutation is usually found before malignant transformation of colorectal neoplasms. Therefore CD44 upregulation is an earlier marker of colonic epithelial transformation than ras mutations.

Colonic adenomas and carcinomas display isoforms containing different sequences encoded by exons v3-v10, which are not encountered at a high level in normal colonic epithelium.[100 101] Furthermore, in adenomatous polyps the presence of these atypical variants correlates with histologically identified areas of dysplasia. At the mRNA level, RT-PCR analysis of transcripts show that there is a greater number of isoforms containing v8-v10 sequences in colon carcinoma than in normal autologous colonic mucosa.[102] The same change can also be visualized by *in situ* hybridization and northern blot experiments (Figure 2).

Immunohistochemical investigations with antibodies specific for discrete variant exon products have documented the pattern of CD44 isoform expression in colon cancer.[97 98] The isoforms containing sequences encoded by alternatively spliced exons v5 and v6 can be traced as early as in small adenomas (diameter < 1cm). In particular, v5 is strongly positive in almost all adenomas, whereas v6 lags behind, being positive in 17% of early

adenomatous lesions and reaching 82% of positivity in advanced carcinomas (Dukes C or D). The expression of v6-containing CD44 isoform in colorectal carcinoma is illustrated in figure 3. In contrast to v5, v6 expression correlates positively with the grade of dysplasia in premalignant tumors. Overall, the acquisition of exon v5 expression occurs early in colorectal tumor development. CD44 isoforms containing v6 appear later, characterize a large subset of neoplasms, probably correspond chronologically to the p53 mutations and lead to a growth advantage of positive tumor cell subclones.[98] Antibodies against epitopes encoded by exon v7 and v8-v10 react strongly with neoplastic cells of adenomas and carcinomas. No products of exon v3 are detected, either in normal or neoplastic colonic epithelium.[97]

Divergent conclusions have been reached regarding a potential relationship between variant CD44 expression and the prognosis of patients with colorectal carcinoma. Protein expression of standard and variant isoforms of CD44 correlates with a poor prognosis in colon cancer as shown by an almost 3 fold deterioration in 5 year survival for patients who have an overexpression of CD44 in their tumor, confirming an earlier report.[103 104] In a retrospective analysis the expression of v6-containing CD44 variants, assessed semiquantitatively, emerged as an adverse prognostic factor independent of Dukes stage.[105]

A prospective study of a larger group of patients reported slightly different results.[106] Although v6 is definitely upregulated as early as in adenomas, its overexpression could not be established as an independent outcome-related parameter. Furthermore, there is a striking down-regulation of v6 in metastatic lesions (i.e. only 17% remain weakly positive, compared with 66% being positive among metastasizing primary tumors). Recently a similar phenomenon was reported by Sugino *et al.*, who demonstrated that the overexpression of both standard and variant CD44 mRNAs and proteins is less prominent in those bladder cancers which are more advanced both in terms of grading and staging.[107] It is therefore possible that, at least in some neoplasms, various CD44 isoforms play only a transient role in progression towards a more malignant phenotype. Alternatively, deep

invasion which is usually achieved by smaller clumps of tumor cells or even single cells might be less dependent on CD44 mediated adhesion.

Investigating protein expression with the help of exon specific antibodies has the main advantage of relating CD44 expression to individual tumor cells in colorectal cancers and is less helpful in defining the splicing pattern. Equally significant is the knowledge of which combinations of CD44 mRNA variant transcripts are expressed in these tumors. This information can be gained from RT-PCR studies.[100 108 109] This approach gives information mostly on tumor cells, although a minor proportion of non-tumor cells are also present in the tumor extracts. Using such a method, it is possible to distinguish patients with a relatively good prognosis (65% survival at 5 years) who have tumors with mostly standard CD44 transcripts, as opposed to patients whose tumors have a high content of CD44 v8-v10 transcripts, defining a 20% 5 year survival.[100] Furthermore high expression of v6 and v8-v10 transcripts in colorectal cancer correlates with the presence of liver metastases.[108 110]

Detailed topographic data on localization of various CD44 mRNAs in colon cancer specimens have recently been reported by a group at the University of Oxford.[111 112] Radioactive *in situ* hybridization with probes specific for variant or standard CD44 allowed them to identify neoplastic but not stromal cells as a source of abundant variant CD44 transcripts. In the latter study *in situ* hybridization was successfully applied to paraffin-embedded specimens, despite low abundance of CD44 message at the mRNA level.

The presence of several species of CD44 transcripts in colonic tumor extracts suggests that tumor cells have dysregulated splicing mechanisms as compared with untransformed epithelial cells. Supporting this notion is the observation of a retained intron in the CD44 transcripts of colon carcinoma.[113] The retained intron, located between v4 and v5, has been originally described in a bladder cancer cell line and clinical specimens of bladder carcinomas. It is found in 77% of bladder carcinoma specimens and proved to be specific as it is not detected in 95% of the control cases.[114] The authors of this study reported the failure of several other CD44 intronic sequences to be edited out in purified

fraction of cytoplasmic mRNA from bladder malignant tumors. However, their subsequent work demonstrated that introns of CD44 mRNAs can also be retained in cytoplasm of some squamous non-neoplastic epithelia, whereas in glandular epithelia this phenomenon is confined to neoplastic cells.[115] Analysis encompassing 15 colon carcinoma samples, autologous normal mucosa specimens and sections of colon mucosa from 12 cases of inflammatory bowel disease showed that cytoplasmic mRNAs containing the whole v4/v5 intron sequence can be detected in all cancer specimens, being absent in all other samples.[115] Moreover, these investigations open perspectives of a new promising colon cancer screening method since intron v4/v5 can be detected in 73% of stool specimens from cancer patients.[116]

Analysis of genomic DNA clones excludes mutations at the exon/intron boundaries as a cause of splicing defects and the mechanism of this puzzling anomaly of mRNA processing still remains unexplained.[114]

The concentration of soluble CD44 increases in the serum of colorectal cancer patients, usually exceeding the normal value by a factor of 10 and returning to normal after curative resection.[36] Thus it can be assayed as a sensitive indicator of tumor burden useful in monitoring the effect of therapy, rather than in initial diagnosis. There is no indication however that serum CD44 could be more sensitive or more specific than serum carcinoembryonic antigen.

It is relevant to ask whether a specific CD44 isoform has a promoting or a suppressing effect on colon cancer, an issue that has been addressed with recent experiments using nude mice xenotransplanted with manipulated cell lines.[109,117] Expression of standard CD44 in colon cancer cells that do not normally express it, leads to a markedly diminished tumorigenicity; this reduction requires an intact cytoplasmic domain. At the same time the cells adhere more strongly to hyaluronan, a function typically associated with the standard isoform of CD44. Taken together, these observations would suggest that the standard isoform of CD44 might have tumor suppressor properties. This suggestion cannot be generalized however, as different human tumors have a different biology in this respect.

Indeed, comparable experiments performed with a melanoma cell line show that the standard isoform of CD44 promotes tumorigenicity in non-epithelial neoplasms.[95]

The exact mechanisms whereby CD44 isoforms containing alternatively spliced exons increase the malignant potential of colon carcinoma cells remains unclear. It is possible that blood group H antigen, which can form a complex with the v6 exon of CD44, participates in tumorigenicity. In a rat colon adenocarcinoma cell line the same antigen is responsible for high tumorigenicity, whereas inhibition of fucosylation not only abrogates its presentation, but also markedly reduces tumor growth in syngeneic rats.[18] Thus once again the way CD44 radically modifies cell behavior seems to be dependent on the unique pattern of glycosylation, itself determined by alternative splicing. It is established that the expression of this carbohydrate antigen correlates negatively with the prognosis of human colorectal cancer.[118]

Gastric carcinoma

Expression of CD44 in the mucosa of normal human stomach resembles the normal pattern of the intestinal epithelium: standard CD44 is present in most epithelial cells, with the exception of those situated in the apical parts of the glands. Isoforms containing products of exon v9 are confined to the basal parts of the glands.[25] To some extent the analogies in CD44 expression also apply to the neoplasms in these two organs. The carcinomatous gastric mucosa is characterized by a relative predominance of transcripts derived from the "epithelial" isoform (CD44E), containing sequence v8-v10, over the standard transcript.[119] Additionally this transcript expression pattern correlates positively with adverse features such as depth of invasion, blood and lymphatic vessel invasion, and presence of lymph node and hepatic metastases.[119,120]

Gastric carcinomas also express the products of v5 and v6 exons with a specificity that relates to the histological subtype: the intestinal type of gastric adenocarcinoma displays v5 and v6 containing CD44 isoforms, whereas the diffuse type of tumor expresses v5 but not v6.[121] This specificity in CD44 variant expression for the intestinal type of

gastric carcinoma is highly consistent with the presence of both v5 and v6 exon products in intestinal metaplasia of the stomach, a change considered putatively premalignant.

Thyroid carcinoma

The pattern of CD44 expression corresponds to the histological features in two most frequent types of thyroid carcinoma, follicular and papillary. Overall expression of CD44 is stronger than in non-neoplastic thyroid tissue for both types of carcinoma. Almost all papillary tumors overexpress the v6 isoform of CD44 as opposed to nearly all follicular neoplasms which are negative for this marker.[122] Considering that papillary carcinomas of the thyroid have a marked tendency to metastasize to lymph nodes, it appears logical to suggest that the v6 variant of CD44 is involved in this metastatic homing.

Other carcinomas

The detailed characterization of CD44 in human colon cancer has been accompanied by studies of other human carcinomas: breast[123-125], pancreas[28 126-128], endometrium[129], cervix[130 131], ovary[35 132], nasopharynx[133], lungs[134], gastrointestinal neuroendocrine system[135] and others[33]. The overall picture on the role of CD44 in the progression of carcinomas and related (neuroendocrine) neoplasms points to a dysregulation of alternative splicing, resulting in the generation of unusual large transcripts, usually accompanied by the translation of high levels of corresponding protein products. Despite some discrepancies between the studies, the neoplasms exhibiting stronger CD44 expression, and especially those producing atypical CD44 variants tend to behave more aggressively. In particular, the expression of the exon v6 product is often associated with adverse clinical and morphological features, mainly with the lymph node metastasis.

Furthermore, interesting facts related to CD44 expression by carcinomatous cells require additional comments, especially when they do not fit with the simplified picture outlined above.

In the case of breast carcinoma the investigation of clinical significance of v6 expression led to divergent conclusions. In a study of 91 patients with a median follow-up of 35 months, CD44 expression was found to be an indicator of poor prognosis in terms of overall survival.[124] By contrast, a study of a larger cohort (227 patients with median observation time 57 months), expression of none of the variant exons, including v6, was found to correlate with the clinical outcome.[125] However, this report found that v6 correlates positively with tumor grade and steroid receptor status.

The significance of CD44 expression in lung cancer has been studied. In an investigation of lung adenocarcinoma using an antibody that does not distinguish standard from variant CD44 isoforms, a poor prognosis is associated with decreased CD44 expression.[136] Furthermore, in 9 cases of small cell carcinoma of the lung, no CD44 expression was found, which contrasts with the positive reaction of most non-small cell primary lung tumors.[134]

The results of these studies contribute to the complexity of interrelations between CD44 expression and behavior of neoplastic cells: they contrast with most of the analogous research on gastrointestinal tumors, but seem to support the experimental data showing that reintroduction of CD44H into colon carcinoma cells reduces its tumorigenicity.[109]

Besides carcinomas characterized by either positive or negative associations between clinical outcome and expression of CD44, there are also some in which complete data on prognostic significance (as defined by overall survival and recurrence-free survival) are still unknown. Nevertheless, in these tumors, CD44 expression has been compared to specific pathological and clinical features. For instance in ovarian serous carcinoma there is a link between the CD44 on the surface of cancer cells and the way this neoplasm spreads in the peritoneal cavity.[137] CD44 negative ovarian carcinomas tend to form ascites, whereas CD44 positive ones grow solid peritoneal implants. Furthermore, the adhesion of ovarian adenocarcinoma cells to mesothelium is hyaluronan and CD44 dependent.

Finally, CD44 expression may be helpful in differential diagnosis, since kidney oncocytomas have been demonstrated to be invariably CD44 negative, whereas

chromophobe renal carcinomas tend to express CD44 including the variant isoforms.[138] In clear cell and chromophilic carcinomas analyzed in this study, a positive correlation was shown between progression from grade G1 to G3 and expression of exons v6 and 9 as well as standard CD44.

Other neoplasms.

In contrast to carcinomas and lymphomas, data on CD44 expression in other neoplasms are rather scant. The expression of CD44 by mesenchymal and neural tissues is usually confined to the standard form of the molecule. In the few neoplasms in which CD44 production was analyzed, this pattern of expression is generally retained, the standard form being the only, if any, detectable isoform.

The observations concerning neuroblastoma are particularly interesting. CD44 is downregulated in advanced neuroblastomas (stage IV), whereas the earlier (I, II, III) or advanced but prognostically favorable stages are characterized by tumor cells maintaining their ability to synthesize the standard form of CD44.[139-141] Approximately half of the stage IV tumors diagnosed lose CD44 expression, resembling the phenotype typical for early neural crest cells.[141] Furthermore, preservation of CD44 is inversely correlated with N-myc amplification, these 2 parameters being the only two statistically independent prognostic factors in multivariate analysis.[142] Northern blot as well as immunohistochemistry with antibodies specific for variant exons products demonstrate that the standard isoform of CD44 is the only one present in neuroblastomas.[140] However, it is still unclear how the suppression of CD44 expression modifies the properties of neuroblastoma cells.

In contrast to medulloblastomas, glioblastomas tend to show a completely different phenotype. These invariably highly malignant neoplasms show strong immunostaining with anti CD44 antibodies recognizing the standard form of CD44.[143] Complementary studies indicate that the expression of variant CD44 isoforms can be traced in many glioblastomas, but is usually confined to a few neoplastic cells.[144] Highly malignant gliomas tend to

contain clusters of v5 positive cells, whereas v6 positive foci could be found in many gliomas irrespective of the grade.[145]

Peripheral schwannomas are characterized by a high expression of CD44 variants with sequences encoded by exon v5, and v7 to v10.[146] Meningiomas are CD44 negative.[143]

Conclusions

In the last few years basic and clinical research on CD44 have led to a set of challenging new concepts. First described as a molecule mostly involved in lymphocyte homing, it is now apparent that CD44 exerts major roles in the biology of cancer cells. Although early historic evidence implicated CD44 in the determination of metastasis, it is clear that its role in tumor progression occurs at a much earlier stage of the transformation process. The pleiotropic expression of CD44 in many non-neoplastic and neoplastic cells is not an indication that it is unrelated to tumor progression. In fact the knowledge of the genomic DNA structure and alternative splicing pattern of CD44 have clearly identified that it is not one but a family of proteins and that discrete isoforms are expressed and regulated at various stages of malignant transformation. The clinico-pathological counterpart of this differential expression of specific isoforms can be summarized by stating that for most, but not all cancers, overexpression of discrete species of CD44 correlates with tumor aggressiveness. A challenging area of research is to define what cellular functions are associated with the various isoforms of CD44 that are overexpressed in cancer. How cell adhesion properties might be gained or lost by these isoforms is an unresolved issue, as well as adhesion to the various ligands found in peritumoral stroma.

Acknowledgements

We thank Dr Sean Moore for a critical analysis of this review, Lam Leduy, Diane McClure and Roya Haghighat for their contribution in the figures and Dr Ursula Günthert for CD44 helpful discussion, and for providing antibodies and probes.

Table 1: CD44 variant expression in human cancer.

Human tumor	CD44 species* expression	Relevance to tumor progression	References
Pancreatic carcinoma	v6	?	Gansauge 1995
Non-Hodgkin Lymphoma	v3, v6, v9	identifies poor prognosis	Stauder 1995
Melanoma	v5	biologically more aggressive	Manten-Horst 1995
Colonic carcinoma	v5, v6	correlates with malignant transformation	Mulder 1995 Finke 1995
Gastric carcinoma	v8-v10	correlates with metastases	Miwa 1996 Yamaguchi 1995
Thyroid carcinoma (papillary type)	v6	correlates with lymph node metastases	Figge 1994
Lung adenocarcinoma	N.I.	loss of overall CD44 expression correlates with poor prognosis	Clarke 1995
Neuroblastoma	standard CD44	loss of overall CD44 expression correlates with poor prognosis	Combaret 1995

*CD44 isoform in this listing are either undetectable in autologous normal cells from the same organ or are present at low concentration and markedly overexpressed in malignant tumors.

N.I. = not investigated

Figure legends

Figure 1: **CD44 structure**

Bold faced regions in the distal extracellular domain identify the hyaluronan binding sequences. Sequences from up to 10 alternatively spliced exons (v1-v10) can be inserted in the proximal extracellular domain. In humans the v1 exon contains a stop codon and is not expressed. The cytoplasmic domain interacts with the actin filaments through ankyrin (A) or members of the ERM (E = ezrin, R = radixin, M = moesin) family.

●●● chondroitin sulfate; **** heparan sulfate; ooooo blood group H antigen

Figure 2: **Selective expression of alternatively spliced variant CD44 in carcinoma**

Northern blot analysis of 2 cases of colon carcinoma is illustrated in the left panel. The membrane was probed with a cDNA consisting of the CD44 v8-v10 sequence. Beta actin load controls are on the lower left panel. Three transcripts with sizes of 2.0, 2.6, and 5.1 kb are detected in the tumor (T) and not in the autologous normal (N) mucosa. The arrowheads indicate the position of 18S and 28S RNA. The right panel illustrates *in situ* hybridization of a colon carcinoma tissue section with an ^{35}S labelled CD44 v8-v10 riboprobe. It shows a strong signal in the neoplastic glands and no detectable signal in the stromal cells (Magnification X140).

Figure 3: **Overexpression and basal distribution of CD44 in carcinoma**

Cryostat section of human colon carcinoma was immunostained for the expression of a variant CD44 isoform containing the product of v6 exon. There is a strong expression of CD44 at the basal membrane of the tumor cells facing the extracellular matrix (arrows). Tumor cells in some parts of the neoplastic glands also express CD44 on their lateral membrane. No v6-containing CD44 isoforms are detected when the normal mucosa is immunostained. (Magnification X140, FW11-9 monoclonal antibody, immunoperoxidase staining of frozen section, light hematoxylin counterstaining).

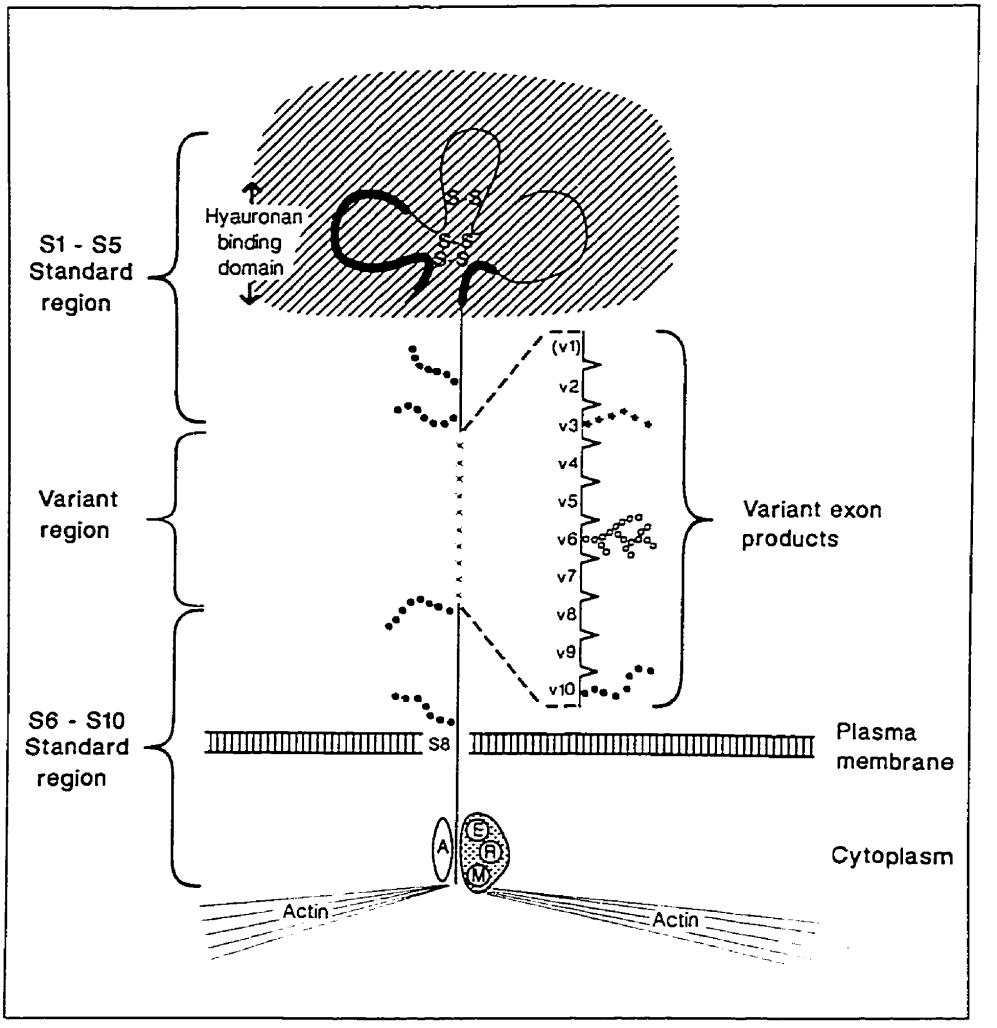

S1 - S5
Standard
region
Hyauronan
binding
domain
S-S
S-S
S-S
Variant
region
(v1)
v2
v3
v4
v5
v6
v7
v8
v9
v10
Variant exon
products
S6 - S10
Standard
region
S8
Plasma
membrane
A
E
R
M
Cytoplasm
Actin
Actin

Fig. 1.

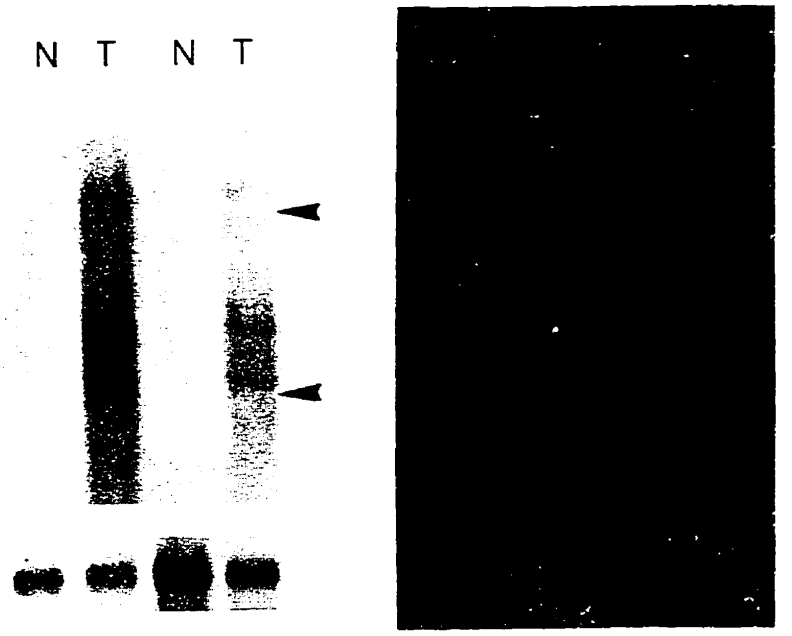


Fig. 2.

Fig. 3.

References

1 Cobbold S. Human leukocyte differentiation antigens: monoclonal antibody computer databases as a tool for the future. *Mol Cell Probes* 1987;**1**:61-72.

2 Dalchau R, Kirkley J, Fabre JW. Monoclonal antibody to a human brain-granulocyte-T lymphocyte antigen probably homologous to the W 3/13 antigen of the rat. *Eur J Immunol* 1980;**10**:745-9.

3 Haynes BF, Liao HX, Patton KL. The transmembrane hyaluronate receptor (CD44): multiple functions, multiple forms. *Cancer Cells* 1991;**3**:347-50.

4 Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. *Adv Immunol* 1993;**54**:271-335.

5 Goodfellow PN, Banting G, Wiles MV, Tunnacliffe A, Parkar M, Solomon E, et al. The gene, MIC4, which controls expression of the antigen defined by monoclonal antibody F10.44.2, is on human chromosome 11. *Eur J Immunol* 1982;**12**:659-63.

6 Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* 1992;**89**:12160-4.

7 Tölg C, Hofmann M, Herrlich P, Ponta H. Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res* 1993;**21**:1225-9.

8 Screaton GR, Bell MV, Bell JI, Jackson DG. The identification of a new alternative exon with highly restricted tissue expression in transcripts encoding the mouse Pgp-1 (CD44) homing receptor. Comparison of all 10 variable exons between mouse, human, and rat. *J Biol Chem* 1993;**268**:12235-8.

9 Sherman L, Sleeman J, Herrlich P, Ponta H. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Curr Opin Cell Biol* 1994;**6**:726-33.

10 Cooper DL, Dougherty GJ. To metastasize or not? Selection of CD44 splice sites. *Nat Med* 1995;**1**:635-7.

11 Kugelman LC, Ganguly S, Haggerty JG, Weissman SM, Milstone LM. The core protein of epican, a heparan sulfate proteoglycan on keratinocytes, is an alternative form of CD44. *J Invest Dermatol* 1992;**99**:886-91.

12 Stamenkovic I, Amiot M, Pesando JM, Seed B. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell* 1989;**56**:1057-62.

13 Nottenburg C, Rees G, St. John T. Isolation of mouse CD44 cDNA: structural features are distinct from the primate cDNA. *Proc Natl Acad Sci USA* 1989;**86**:8521-5.

14 Yang B, Yang BL, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J* 1994;**13**:286-96.

15 Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A. Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J Cell Biol* 1993;**122**:257-64.

16 Liao HX, Lee DM, Levesque MC, Haynes BF. N-terminal and central regions of the human CD44 extracellular domain participate in cell surface hyaluronan binding. *J Immunol* 1995;**155**:3938-45.

17 Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, Whittle N. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J Cell Biol* 1995;**128**:673-85.

18 Labarrière N, Piau JP, Otry C, Denis M, Lustenberger P, Meflah K, et al. H blood group antigen carried by CD44V modulates tumorigenicity of rat colon carcinoma cells. *Cancer Res* 1994;**54**:6275-81.

19 Goldstein LA, Butcher EC. Identification of mRNA that encodes an alternative form of H-CAM(CD44) in lymphoid and nonlymphoid tissues. *Immunogenetics* 1990;**32**:389-97.

20 Lokeshwar VB, Bourguignon LY. The lymphoma transmembrane glycoprotein GP85 (CD44) is a novel guanine nucleotide-binding protein which regulates GP85 (CD44)-ankyrin interaction. *J Biol Chem* 1992;**267**:22073-8.

21 Lokeshwar VB, Fregien N, Bourguignon LY. Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *J Cell Biol* 1994;**126**:1099-109.

22 Bennett KL, Modrell B, Greenfield B, Bartolazzi A, Stamenkovic I, Peach R, et al. Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. *J Cell Biol* 1995;**131**:1623-33.

23 Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J* 1992;**6**:861-70.

24 Stamenkovic I, Aruffo A, Amiot M, Seed B. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J* 1991;**10**:343-8.

25 Terpe HJ, Stark H, Prehm P, Günthert U. CD44 variant isoforms are preferentially expressed in basal epithelial of non-malignant human fetal and adult tissues. *Histochemistry* 1994;**101**:79-89.

26 Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Günthert U. Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol* 1994;**124**:71-82.

27 Iida N, Bourguignon LY. New CD44 splice variants associated with human breast cancers. *J Cell Physiol* 1995;**162**:127-33.

28 Gansauge F, Gansauge S, Zobywalski A, Scharnweber C, Link KH, Nussler AK, et al. Differential expression of CD44 splice variants in human pancreatic adenocarcinoma and in normal pancreas. *Cancer Res* 1995;**55**:5499-503.

29 Hong RL, Pu YS, Chu JS, Lee WJ, Chen YC, Wu CW. Correlation of expression of CD44 isoforms and E-cadherin with differentiation in human urothelial cell lines and transitional cell carcinoma. *Cancer Lett* 1995;**89**:81-7.

30 Southgate J, Trejdosiewicz LK, Smith B, Selby PJ. Patterns of splice variant CD44 expression by normal human urothelium in situ and in vitro and by bladder-carcinoma cell lines. *Int J Cancer* 1995;**62**:449-56.

31 Neame SJ, Isacke CM. Phosphorylation of CD44 in vivo requires both Ser323 and Ser325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells. *EMBO J* 1992;**11**:4733-8.

32 Isacke CM. The role of the cytoplasmic domain in regulating CD44 function. *J Cell Sci* 1994;**107**:2353-9.

33 Herrlich P, Zoller M, Pals ST, Ponta H. CD44 splice variants: metastases meet lymphocytes. *Immunol Today* 1993;**14**:395-9.

34 Hofmann M, Rudy W, Zoller M, Tölg C, Ponta H, Herrlich P, et al. CD44 splice variants confer metastatic behavior in rats: homologous sequences are expressed in human tumor cell lines. *Cancer Res* 1991;**51**:5292-7.

35 Sliutz G, Tempfer C, Winkler S, Kohlberger P, Reinthaller A, Kainz C. Immunohistochemical and serological evaluation of CD44 splice variants in human ovarian cancer. *Br J Cancer* 1995;**72**:1494-7.

36 Guo YJ, Liu G, Wang X, Jin D, Wu M, Ma J, et al. Potential use of soluble CD44 in serum as indicator of tumor burden and metastasis in patients with gastric or colon cancer. *Cancer Res* 1994;**54**:422-6.

37 Underhill CB, Chi-Rosso G, Toole BP. Effects of detergent solubilization on the hyaluronate-binding protein from membranes of simian virus 40-transformed 3T3 cells. *J Biol Chem* 1983;**258**:8086-91.

38 Underhill CB, Thurn AL, Lacy BE. Characterization and identification of the hyaluronate binding site from membranes of SV-3T3 cells. *J Biol Chem* 1985;**260**:8128-33.

39 Underhill CB, Green SJ, Comoglio PM, Tarone G. The hyaluronate receptor is identical to a glycoprotein of Mr 85.000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. *J Biol Chem* 1987;**262**:13142-6.

40 Miyake K, Underhill CB, Lesley J, Kincade PW. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J Exp Med* 1990;**172**:69-75.

41 Zheng Z, Katoh S, He Q, Oritani K, Miyake K, Lesley J, et al. Monoclonal antibodies to CD44 and their influence on hyaluronan recognition. *J Cell Biol* 1995;**130**:485-95.

42 Koshiishi I, Shizari M, Underhill CB. CD44 can mediate the adhesion of platelets to hyaluronan. *Blood* 1994;**84**:390-6.

43 Milstone LM, Hough-Monroe L, Kugelman LC, Bender JR, Haggerty JG. Epican, a heparan/chondroitin sulfate proteoglycan form of CD44, mediates cell-cell adhesion. *J Cell Sci* 1994;**107**:3183-90.

44 Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (ETA-1). *Science* 1996;**271**:509-12.

45 Underhill C. CD44: the hyaluronan receptor. *J Cell Sci* 1992;**103**:293-8.

46 Thomas L, Byers HR, Vink J, Stamenkovic I. CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J Cell Biol* 1992;**118**:971-7.

47 He Q, Lesley J, Hyman R, Ishihara K, Kincade PW. Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. *J Cell Biol* 1992;**119**:1711-19.

48 Dougherty GJ, Cooper DL, Memory JF, Chiu RK. Ligand binding specificity of alternatively spliced CD44 isoforms. Recognition and binding of hyaluronan by CD44R1. *J Biol Chem* 1994;**269**:9074-8.

49 Droll A, Dougherty ST, Chiu RK, Dirks JF, McBride WH, Cooper DL, et al. Adhesive interactions between alternatively spliced CD44 isoforms. *J Biol Chem* 1995;**270**:11567-73.

50 Lokeshwar VB, Bourguignon LY. Post-translational protein modification and expression of ankyrin-binding site(s) in GP85 (Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biosynthesis. *J Biol Chem* 1991;**266**:17983-9.

51 Bartolazzi A, Jackson D, Bennett K, Aruffo A, Dickinson R, Shields J, et al. Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. *J Cell Sci* 1995;**108**:1723-33.

52 Lesley J, He Q, Miyake K, Hamann A, Hyman R, Kincade PW. Requirements for hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody. *J Exp Med* 1992;**175**:257-66.

53 Lesley J, Schulte R, Hyman R. Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *Exp Cell Res* 1990;**187**:224-33.

54 Laurent TC, Fraser JR. Hyaluronan. *FASEB J* 1992;**6**:2397-404.

55 Culty M, Shizari M, Thompson EW, Underhill CB. Binding and degradation of hyaluronan by human breast cancer cell lines expressing different forms of CD44: correlation with invasive potential. *J Cell Physiol* 1994;**160**:275-86.

56 Knudson CB, Knudson W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J* 1993;**7**:1233-41.

57 Ruiz P, Schwarzler C, Günthert U. CD44 isoforms during differentiation and development. *Bioessays* 1995;**17**:17-24.

58 Bourguignon LY, Lokeshwar VB, Chen X, Kerrick WG. Hyaluronic acid-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J Immunol* 1993;**151**:6634-44.

59 Jalkanen S, Jalkanen M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol* 1992;**116**:817-25.

60 Ishii S, Ford R, Thomas P, Nachman A, Steele G Jr, Jessup JM. CD44 participates in the adhesion of human colorectal carcinoma cells to laminin and type IV collagen. *Surg Oncol* 1993;**2**:255-64.

61 Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K, et al. A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *J Biol Chem* 1995;**270**:7437-44.

62 Bennett KL, Jackson DG, Simon JC, Tanczos E, Peach R, Modrell B, et al. CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. *J Cell Biol* 1995;**128**:687-98.

63 Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. *FASEB J* 1993;**7**:1475-82.

64 Bautista DS, Xuan JW, Hota C, Chambers AF, Harris JF. Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin (1994). *J Biol Chem* 1994;**269**:23280-5.

65 Xuan JW, Hota C, Chambers AF. Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. *J Cell Biochem* 1994;**54**:247-55.

66 Hijiya N, Setoguchi M, Matsuura K, Higuchi Y, Akizuki S, Yamamoto S. Cloning and characterization of the human osteopontin gene and its promoter. *Biochem J* 1994;**303**:255-62.

67 Senger DR, Brown LF, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Osteopontin at the tumor/host interface. Functional regulation by thrombin-cleavage and consequences for cell adhesion. *Ann N Y Acad Sci* 1995;**760**:83-100.

68 Jalkanen ST, Bargatze RF, Herron LR, Butcher EC. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur J Immunol* 1986;**16**:1195-202.

69 Arch R, Wirth K, Hofmann M, Ponta H, Matzku S, Herrlich P, et al. Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science* 1992;**257**:682-5.

70 Koopman G, Heider KH, Horst E, Adolf GR, van den Berg F, Ponta H, et al. Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med* 1993;**177**:897-904.

71 de los Toyos J, Jalkanen S, Butcher EC. Flow cytometric analysis of the Hermes homing-associated antigen on human lymphocyte subsets. *Blood* 1989;**74**:751-60.

72 O'Neill HC. Antibody which defines a subset of bone marrow cells that can migrate to thymus. *Immunology* 1989;**68**:59-65.

73 Horst E, Meijer CJ, Radaskiewicz T, van Dongen JJ, Pieters R, Figdor CG, et al. Expression of a human homing receptor (CD44) in lymphoid malignancies and related stages of lymphoid development. *Leukemia* 1990;**4**:383-9.

74 Miyake K, Medina KL, Hayashi S, Ono S, Hamaoka T, Kincade PW. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J. Exp Med* 1990;**171**:477-88.

75 Bourguignon LY, Walker G, Suchard SJ, Balazovich K. A lymphoma plasma membrane-associated protein with ankyrin-like properties. *J Cell Biol* 1986;**102**:2115-24.

76 Kalomiris EL, Bourguignon LY. Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J Cell Biol* 1988;**106**:319-27.

77 Perschl A, Lesley J, English N, Trowbridge I, Hyman R. Role of CD44 cytoplasmic domain in hyaluronan binding. *Eur J Immunol* 1995;**25**:495-501.

78 Murakami S, Shimabukuro Y, Miki Y, Saho T, Hino E, Kasai D, et al. Inducible binding of human lymphocytes to hyaluronate via CD44 does not require cytoskeleton association but does require new protein synthesis. *J Immunol* 1994;**152**:467-77.

79 Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 1994;**126**:391-401.

80 Sato N, Funayama N, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites. *J Cell Sci* 1992;**103**:131-43.

81 Günthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, et al. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991;**65**:13-24.

82 Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, et al. Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 1993;**177**:443-55.

83 Matsumura Y, Tarin D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 1992;**340**:1053-8.

84 Sy MS, Guo YJ, Stamenkovic I. Distinct effects of two CD44 isoforms on tumor growth in vivo. *J Exp Med* 1991;**174**:859-66.

85 Fujiwara K, Yoshino T, Miyake K, Ohara N, Akagi T. Expression of a lymphocyte adhesion molecule (CD44) in malignant lymphomas: relevance to primary site, histological subtype and clinical stage. *Acta Med Okayama* 1993;**47**:215-22.

86 Joensuu H, Ristamaki R, Klemi PJ, Jalkanen S. Lymphocyte homing receptor (CD44) expression is associated with poor prognosis in gastrointestinal lymphoma. *Br J Cancer* 1993;**68**:428-32.

87 Salles G, Zain M, Jiang WM, Boussiotis VA, Shipp MA. Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas: characterization and comparison with normal activated B cells and epithelial malignancies. *Blood* 1993;**82**:3539-47.

88 Terpe HJ, Koopmann R, Imhof BA, Günthert U. Expression of integrins and CD44 isoforms in non-Hodgkin's lymphomas: CD44 variant isoforms are preferentially expressed in high-grade malignant lymphomas. *J Pathol* 1994;**174**:89-100.

89 Stauder R, Eisterer W, Thaler J, Günthert U. CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. *Blood* 1995;**85**:2885-99.

90 Driessens MHE, Stroeken PJM, Erena NFR, Vandervalk MA, Vanrijthoven EAM, Roos E. Targeted disruption of CD44 in mday-d2 lymphosarcoma cells has no effect on subcutaneous growth or metastatic capacity. *J Cell Biol* 1995;**131**:1849-55.

91 Ristamaki R, Joensuu H, Salmi M, Jalkanen S. Serum CD44 in malignant lymphoma: an association with treatment response. *Blood* 1994;**84**:238-43.

92 Manten-Horst E, Danen EH, Smit L, Snoek M, Le Poole IC, Van Muijen GN, et al. Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential. *Int J Cancer* 1995;**64**:182-8.

93 Seiter S, Schadendorf D, Herrmann K, Schneider M, Rosel M, Arch R, et al. Expression of CD44 variant isoforms in malignant melanoma. *Clin Canc Res* 1996;**2**:447-56.

94 Van Muijen GN, Danen EH, Veerkamp JH, Ruiter DJ, Lesley J, van den Heuvel LP. Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity. *Int J Cancer* 1995;**61**:241-8.

95 Bartolazzi A, Peach R, Aruffo A, Stamenkovic I. Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. *J Exp Med* 1994;**180**:53-66.

96 Birch M, Mitchell S, Hart IR. Isolation and characterization of human melanoma cell variants expressing high and low levels of CD44. *Cancer Res* 1991;**51**:6660-7.

97 Wielenga VJ, Heider KH, Offerhaus GJ, Adolf GR, van den Berg FM, Ponta H, et al. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. *Cancer Res* 1993;**53**:4754-6.

98 Mulder JW, Wielenga VJ, Polak MM, van den Berg FM, Adolf GR, Herrlich P, et al. Expression of mutant p53 protein and CD44 variant proteins in colorectal tumorigenesis. *Gut* 1995;**36**:76-80.

99 Kim H, Yang XL, Rosada C, Hamilton SR, August JT. CD44 expression in colorectal adenomas is an early event occurring prior to K-ras and p53 gene mutation. *Arch Biochem Biophys* 1994;**310**:504-7.

100 Finn L, Dougherty G, Finley G, Meisler A, Becich M, Cooper DL. Alternative splicing of CD44 pre-mRNA in human colorectal tumors. *Biochem Biophys Res Commun* 1994;**200**:1015-22.

101 Heider KH, Hofmann M, Hors E, van den Berg F, Ponta H, Herrlich P, et al. A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J Cell Biol* 1993;**120**:227-33.

102 Tanabe KK, Ellis LM, Saya H. Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. *Lancet* 1993;**341**:725-6.

103 Ichikawa W. Positive relationship between expression of CD44 and hepatic metastases in colorectal cancer. *Pathobiology* 1994;**62**:172-9.

104 Abbasi AM, Chester KA, Talbot IC, Macpherson AS, Boxer G, Forbes A, et al. CD44 is associated with proliferation in normal and neoplastic human colorectal epithelial cells. *Eur J Cancer* 1993;**29A**:1995-2002.

105 Mulder JW, Kruyt PM, Sewnath M, Oosting J, Seldenrijk CA, Weidema WF, et al. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1994;**344**:1470-2.

106 Finke LH, Terpe HJ, Zorb C, Haensch W, Schlag PM. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1995;**345**:583.

107 Sugino T, Gorham H, Yoshida K, Bolodeoku J, Nargund V, Cranston D, et al. Progressive loss of CD44 gene expression in invasive bladder cancer. *Am J Pathol* 1996;**149**:873-82.

108 Takeuchi K, Yamaguchi A, Urano T, Goi T, Nakagawara G, Shiku H. Expression of CD44 variant exons 8-10 in colorectal cancer and its relationship to metastasis. *Jpn J Cancer Res* 1995;**86**:292-7.

109 Tanabe KK, Stamenkovic I, Cutler M, Takahashi K. Restoration of CD44H expression in colon carcinomas reduces tumorigenicity. *Ann Surg* 1995;**222**:493-501.

110 Rodriguez C, Monges G, Rouanet P, Dutrillaux B, Lefrancois D, Theillet C. CD44 expression patterns in breast and colon tumors: a PCR-based study of splice variants. *Int J Cancer* 1995;**64**:347-54.

111 Gorham H, Sugino T, Woodman AC, Tarin D. Cellular distribution of CD44 gene transcripts in colorectal carcinomas and in normal colonic mucosa. *J Clin Pathol* 1996;**49**:482-8.

112 Gorham H, Sugino T, Bolodeoku J, Yoshida K, Goodison S, Tarin D. Distribution of CD44 messenger RNA in archival paraffin wax embedded tumours and normal tissues viewed by in situ hybridisation. *J Clin Pathol: Mol Pathol* 1996;**49**:M147-M150.

113 Yoshida K, Bolodeoku J, Sugino T, Goodison S, Matsumura Y, Warren BF, et al. Abnormal retention of intron 9 in CD44 gene transcripts in human gastrointestinal tumors. *Cancer Res* 1995;**55**:4273-7.

114 Matsumura Y, Sugiyama M, Matsumura S, Hayle AJ, Robinson P, Smith JC, et al. Unusual retention of introns in CD44 gene transcripts in bladder cancer provides new diagnostic and clinical oncological opportunities. *J Pathol* 1995;**177**:11-20.

115 Higashikawa K, Yokozaki H, Ue T, Taniyama K, Ishikawa T, Tarin D, et al. Evaluation of CD44 transcription variants in human digestive tract carcinomas and normal tissues. *Int J Cancer* 1996;**66**:11-7.

116 Yoshida K, Sugino T, Bolodeoku J, Warren BF, Goodison S, Woodman A, et al. Detection of exfoliated carcinoma cells in colonic luminal washings by identification of deranged patterns of expression of the CD44 gene. *J Clin Pathol* 1996;**49**:300-5.

117 Takahashi K, Stamenkovic I, Cutler M, Saya H, Tanabe KK et al. CD44 hyaluronate binding influences growth kinetics and tumorigenicity of human colon carcinomas. *Oncogene* 1995;**11**:2223-32.

118 Miwa T, Watanabe A, Yamada Y, Shino Y, Yamada T, Yamashita J, et al. Progression in gastric carcinoma relative to the ratio of CD44 epithelial variant transcript to CD44 hematopoietic variant transcript. *Cancer* 1996;**77**:25-9.

119 Naitoh H, Yazawa S, Asao T, Nakajima T, Nakamura J, Takenoshita S, et al. The recognition of cancer-associated fucosylated antigens in colorectal cancer by a novel monoclonal antibody, YB-2. *Surg Today* 1994;**24**:382-4.

120 Yamaguchi A, Saito M, Goi T, Iida A, Takeuchi K, Hirose K, et al. Expression of CD44 variant exons 8-10 in gastric cancer. *Jpn J Cancer Res* 1995;**86**:1166-71.

121 Dämmrich J, Vollmers HP, Heider KH, Müller-Hermelink HK. Importance of different CD44v6 expression in human gastric intestinal and diffuse type cancers for metastatic lymphogenic spreading. *J Mol Med* 1995;**73**:395-401.

122 Figge J, del Rosario AD, Gerasimov G, Dedov I, Bronstein M, Troshina K, et al. Preferential expression of the cell adhesion molecule CD44 in papillary thyroid carcinoma. *Exp Mol Pathol* 1994;**61**:203-11.

123 Friedrichs K, Franke F, Lisboa BW, Kügler G, Gille I, Terpe HJ, et al. CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. *Cancer Res* 1995;**55**:5424-33.

124 Kaufmann M, Heider KH, Sinn HP, von Minckwitz G, Ponta H, Herrlich P. CD44 variant exon epitopes in primary breast cancer and length of survival. *Lancet* 1995;**345**:615-19.

125 Sinn HP, Heider KH, Skroch-Angel P, von Minckwitz G, Kaufmann M, Herrlich P, et al. Human mammary carcinomas express homologues of rat metastasis-associated variants of CD44. *Breast Cancer Res Treat* 1995;**36**:307-13.

126 Rall CJ, Rustgi AK. CD44 isoform expression in primary and metastatic pancreatic adenocarcinoma. *Cancer Res* 1995;**55**:1831-5.

127 Takada M, Yamamoto M, Saitoh Y. The significance of CD44 in human pancreatic cancer: I. High expression of CD44 in human pancreatic adenocarcinoma. *Pancreas* 1994;**9**:748-52.

128 Takada M, Yamamoto M, Saitoh Y. The significance of CD44 in human pancreatic cancer: II. The role of CD44 in human pancreatic adenocarcinoma invasion. *Pancreas* 1994;**9**:753-7.

129 Fujita N, Yaegashi N, Ide Y, Sato S, Nakamura M, Ishiwata I, et al. Expression of CD44 in normal human versus tumor endometrial tissues: possible implication of reduced expression of CD44 in lymph-vascular space involvement of cancer cells. *Cancer Res* 1994;**54**:3922-8.

130 Kainz C, Kohlberger P, Tempfer C, Sliutz G, Gitsch G, Reinthaller A, et al. Prognostic value of CD44 splice variants in human stage III cervical cancer. *Eur J Cancer* 1995;**31A**:1706-9.

131 Dall P, Heider KH, Hekele A, von Minckwitz G, Kaufmann M, Ponta H, et al. Surface protein expression and messenger RNA-splicing analysis of CD44 in uterine cervical cancer and normal cervical epithelium. *Cancer Res* 1994;**54**:3337-41.

132 Uhl-Steidl M, Muller-Holzner E, Zeimet AG, Adolf GR, Daxenbichler G, Marth C, et al. Prognostic value of CD44 splice variant expression in ovarian cancer. *Oncology* 1995;**52**:400-6.

133 Brooks L, Niedobitek G, Agathanggelou A, Farrell PJ. The expression of variant CD44 in nasopharyngeal carcinoma is unrelated to expression of LMP-1. *Am J Pathol* 1995;**146**:1102-12.

134 Penno MB, August JT, Baylin SB, Mabry M, Linnoila RI, Lee VS, et al. Expression of CD44 in human lung tumors. *Cancer Res* 1994;**54**:1381-7.

135 Chaudhry A, Gobl A, Eriksson B, Skogseid B, Oberg K. Different splice variants of CD44 are expressed in gastrinomas but not in other subtypes of endocrine pancreatic tumors. *Cancer Res* 1994;**54**:981-6.

136 Clarke MR, Landernau RJ, Resnick NM, Crowley R, Dougherty GJ, Cooper DL, et al. Prognostic significance of CD44 expression in adenocarcinoma of the lung. *J Clin Pathol: Mol Pathol* 1995;**48**:M200-M204.

137 Cannistra SA, Kansas GS, Niloff J, DeFranzo B, Kim Y, Ottensmeier C. Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. *Cancer Res* 1993;**53**:3830-8.

138 Terpe HJ, Storkel S, Zimmer U, Anquez V, Fischer C, Pantel K, et al. Expression of CD44 isoforms in renal cell tumors - positive correlation to tumor differentiation. *Am J Pathol* 1996;**148**:453-63.

139 Shtivelman E, Bishop JM. Expression of CD44 is repressed in neuroblastoma cells. *Mol Cell Biol* 1991;**11**:5446-53.

140 Gross N, Beretta C, Peruisseau G, Jackson D, Simmons D, Beck D. CD44H expression by human neuroblastoma cells: relation to MYCN amplification and lineage differentiation. *Cancer Res* 1994;**54**:4238-42.

141 Combaret V, Coll JL, Favrot MC. Expression of integrin and CD44 adhesion molecules on neuroblastoma: the relation to tumor aggressiveness and embryonic neural-crest differentiation. *Invasion Metastasis* 1994;**14**:156-63.

142 Combaret V, Lasset C, Frappaz D, Bouvier R, Thiesse P, Rebillard AC, et al. Evaluation of CD44 prognostic value in neuroblastoma: comparison with the other prognostic factors. *Eur J Cancer* 1995;**31A**:545-9.

143 Ariza A, Lopez D, Mate JL, Isamat M, Musulen E, Pujol M, et al. Role of CD44 in the invasiveness of glioblastoma multiforme and the noninvasiveness of meningioma - an immunohistochemistry study. *Hum Pathol* 1995;**26**:1144-7.

144 Eibl RH, Pietsch T, Moll J, Skroch-Angel P, Heider KH, Vonammon K, et al. Expression of variant CD44 epitopes in human astrocytic brain tumors. *J Neurooncol* 1995;**26**:165-70.

145 Kaaijk P, Troost D, Morsink F, Keehnen RMJ, Leenstra S, Bosch DA, et al. Expression of CD44 splice variants in human primary brain tumors. *J Neurooncol* 1995;**26**:185-90.

146 Sherman L, Skroch-Angel P, Moll J, Schwechheimer K, Ponta H, Herrlich P. et al. Schwann cell tumors express characteristic patterns of CD44 splice variants. *J Neurooncol* 1995;**26**:171-84.

Changes in CD44 expression during carcinogenesis of the mouse colon

Zbigniew Rudzki, Lam LeDuy and Serge Jothy

from the Department of Pathology, McGill University, Montreal, QC, Canada

running head: ***CD44 in mouse colon cancer***

corresponding author: Serge Jothy M.D., Ph.D.
Present address:
Department of Laboratory Medicine, Sunnybrook Health Sciences Center and University of Toronto,
2075 Bayview Av., Toronto, ON, Canada M4N 3M5
e-mail address: jothy@pathology.lan.mcgill.ca

Keywords: colon cancer, adenoma, mouse, CD44

ABSTRACT

CD44 glycoprotein is the main extracellular receptor for hyaluronic acid. The CD44 gene is composed of 20 exons and encodes for a variety of isoforms generated by alternative splicing of 10 exons. Overexpression of CD44 and appearance of discrete isoforms containing products of variant exons have been implicated in the progression of cancer, including human colon carcinoma. The pattern of CD44 transcript changes during early colorectal carcinogenesis, and their relation to CD44 protein expression remains to be defined under experimental conditions. In the current study we investigated CD44 expression in a murine model of human colon adenoma/carcinoma. Colon tumors were induced in 19 ICR/Ha mice by 1,2-dimethylhydrazine (DMH) injections and CD44 expression was studied by RT-PCR/Southern blot analysis and immunohistochemistry. CD44 transcripts were strongly overexpressed in tumors compared with normal colon. Both neoplastic and normal colon samples exhibited the same species of CD44 transcript representing standard and variant isoforms. Seventy five per cent of neoplasms contained foci of tumor cells expressing CD44 protein, whereas in normal colon the epithelial immunoreactivity was confined to the crypt base. Immunostaining of neoplastic cells was heterogeneous and there was a significant tendency towards the progressive loss of CD44 immunoreactivity in large and invading tumors. It is concluded that early events in murine colorectal carcinogenesis are characterized by a marked global overexpression of standard and variant CD44 transcripts.

INTRODUCTION

The stroma of most malignant tumors contains a large amount of hyaluronic acid (HA), raising the question of whether cancer cells have and use receptors for this ligand. CD44 is one of the main receptors for HA and its involvement in the biology of cancer was derived from a series of experiments that causally linked one of the CD44 isoforms with metastatic potential of a rat pancreatic adenocarcinoma cell line.[1] Data obtained from human malignancies confirmed that the significance of CD44 is not confined to this one particular animal cell line system.[2,3] The picture emerging from those and later studies strongly suggests that particular CD44 isoforms contribute differently to the origin and progression of various malignancies (reviewed in [4]).

CD44 is the product of a single gene, composed of at least 20 exons.[5] The distal part of its extracellular domain is encoded by 5 exons (s1 to s5). It is very highly conserved among species and contains two HA binding motifs.[6] It can be followed by an insert of up to 381 aminoacids resulting from alternative splicing of at least 10 "variant" exons (v1-v10). The remaining standard exons (s6 to s10) encode the short membrane-proximal domain, transmembrane portion of the molecule and its intracytoplasmic tail. The latter can associate with the cytoskeleton via ankyrin[7] and ezrin - moesin - radixin family of proteins.[8] Apart from its well characterized HA binding function, CD44 probably binds chondroitin sulfate, fibronectin, serglycin, and osteopontin and possibly itself (reviewed in [4]).

The shortest CD44 isoform (CD44s, also called the "standard" or "hematopoietic" form), lacking the alternatively spliced insert, is ubiquitously present on the surface of most cells. Variant CD44 isoforms are much more restricted and mostly confined to some epithelial cells and activated lymphocytes. In the majority of human neoplasms, both the CD44 mRNA and proteins are overexpressed, and the expression of new isoforms distinguishes neoplastic cells from their normal counterparts. In human colon cancer progression from adenoma to carcinoma is followed by a gradual derangement of CD44

mRNA splicing with acquisition of atypical isoforms, especially those containing products of exons v5 and v6.[9,10] The significance of strong CD44 immunopositivity as well as expression of v6 encoded epitopes as an independent predictor of colon cancer prognosis is a controversial issue.[10,11]

Several animal models of colon cancer have been widely used to investigate the pathogenesis of its human counterpart. Among them, the 1,2-dimethylhydrazine (DMH) induced murine colon adenoma/carcinoma model has interesting similarities to the human adenoma-carcinoma sequence.[12,13] Similarly to the human colon cancer, the DMH murine model carcinogenesis is affected by diet and by multigenetic factors.[14]

To better understand the critical role played by cell adhesion proteins in a well defined colon carcinogenesis model, we investigated in this study of DMH murine cancer the expression of CD44 transcripts and protein, using RT-PCR and immunohistochemistry.

MATERIALS AND METHODS

Experimental carcinogenesis

Colorectal tumors were induced by injections of 1,2-dimethylhydrazine hydrochloride (DMH) (Sigma Chemical, St Louis, MO) in 17 six-week adult male and female ICR/Ha mice (Roswell Park Cancer Institute, Buffalo, NY). DMH was dissolved in sterile water (4 mg/ml), stabilized with 15 mM EDTA and brought to pH 7 by addition of NaOH. Experimental animals were injected s.c. at the dose of 20 mg/kg body weight once a week for 25 weeks. Nine control animals were injected with sterile water. The mice were maintained according to the regulations of the Canadian Council of on Animal Care in an air-conditioned room on a standard chow and water *ad libitum*. After 25 weeks, all animals were sacrificed by carbon dioxide inhalation and subjected to complete autopsies. At least one colon tumor from each animal as well as a segment of grossly normal colon were snap frozen in liquid nitrogen for RNA extraction. For immunohistochemistry

tumoral and normal colon with tumors were embedded in OCT medium (Miles Scientific, Elkhart, IN) and kept frozen at -80 C.

Immunohistochemistry

Biotinylated anti-mouse monoclonal antibody KM81, directed to a non-polymorphic epitope of CD44 [15] was provided by Cedarlane (Cedarlane Laboratories, Hornby, ON, Canada). In preliminary studies, immunohistochemistry on formaldehyde or ethanol fixed/ paraffin embedded sections using KM81 and three other anti-mouse pan-CD44 monoclonal antibodies (IM7.8.1., IRAWB14.4 and KM201 kindly provided by Dr. Jayne Lesley, Department of Cancer Biology, Salk Institute, San Diego, CA) was negative even after prolonged microwave antigen retrieval. Since all four antibodies gave strong and virtually identical staining patter with frozen sections of both normal and neoplastic mouse colon, all further work was performed using KM81. Cryostat sections were air-dried, endogenous peroxidase activity was quenched with 0.3% methanol solution of H_2O_2 for 30 min and the KM81 antibody (diluted 1:150 in PBS) was applied overnight at 4°C. The positive immunoreaction was visualized with an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Aminoethylcarbazole (Dimension Laboratories Inc, Mississauga, ON, Canada) was used as a chromogen. In negative control experiments the antibody was replaced with normal mouse serum diluted 1:50 in PBS. The immunostaining of Auerbach's neural plexus was used as an internal positive control. All slides were counterstained with hematoxylin.

CD44 expression was graded as - to +++ based on immunostaining intensity. In tumors with heterogenous immunostaining the predominant staining pattern was recorded. The expression of CD44 of intratumoral stromal cells was also scored.

All neoplasms found in frozen sections were classified into 3 grades of differentiation (gr 1: well, gr 2: moderately, and gr 3: poorly differentiated tumors),

according to criteria used for human colon adenocarcinomas.[16] Neoplasms were considered invasive when they breached the muscularis mucosae.

RT-PCR/ Southern blotting of CD44 transcripts

RNA was isolated as previously described.[17] Specimens from 17 pairs of tumoral and autologous normal colon from 11 males and six females as well as colon samples from nine control mice (six males, three females) were analyzed. After overnight isopropanol precipitation, the samples were treated with DNAse I (Boehringer Mannheim, Laval, QC, Canada) and reprecipitated with absolute ethanol. Twelve μg of total RNA was reversely transcribed with random hexamers using Superscript H-RT (Life Technologies, Burlington, ON, Canada), followed by incubation with 0.5 U of RNAse H (Boehringer Mannheim, Laval, QC, Canada). Quality and load of cDNA in each sample was tested by monitoring the expression of a housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) with primers yielding a single 0.45 kb product (Clontech Laboratories, Palo Alto, CA; cat# 5405-1). A Perkin Elmer/Cetus (Norwalk, CT) DNA Thermal Cycler was used and amplification was performed at non-saturating conditions: 25 rounds of denaturation at 95 C for 45 s, annealing at 60 for 1 min and synthesis at 72 C for 1 min; final extension at 72 C for 10 min.

Five percent of the total reverse transcription product in 5 μL of H_20 was used for amplification of CD44 cDNA with the forward (5'TTGAATGTAACCTGCCGCTACGCA 3') and reverse (5'TCGGATCCATGAGTCACAGTGCG 3') primers flanking the alternative splicing site of the published murine CD44 sequence.[18] These primers, complementary to regions within exon s2 and s6, respectively, amplify all CD44 isoforms and give the amplicon of 654 bp with CD44s as a template. PCR was carried out in a 30 μL reaction volume containing 0.17 mM dNTPs, 1.0 mM of $MgSO_4$, 0.1 μg of each primer and 2 U of Vent exo$^-$ DNA polymerase (New England Biolabs, Mississauga, ON, Canada). After an initial 2 min 30 sec of denaturation at 95 C, 30 cycles of denaturation (1 min, 95 C), annealing (1 min, 60 C) and extension (1 min 30 s, 72 C) were performed, followed by

final extension for 10 min at 72 C. PCR amplification of both normal and tumor samples over the wide range of cycles (15-40) revealed that at 30 cycles the reaction does not reach its saturation point (data not shown).

The PCR product was resolved by 1.2% agarose gel electrophoresis and transferred in 0.4 M NaOH to positively charged Hybond-N^+ nylon membranes (Amersham, Oakville, ON, Canada). The membranes were hybridized overnight with an oligonucleotide mouse probe (5' CCATCACGGTTGACAATAGTTATGGTAA 3'), end-labelled with ^{32}P. Variant CD44 isoforms were detected by overnight hybridization with the cDNA probe rA, kindly provided by Dr. Ursula Günthert (Basel Institute for Immunology, Switzerland). The rA probe is derived from a rat adenocarcinoma cell line BSp73ASML, cDNA clone pMeta-1 and recognizes a ~170 bp region within exons v5/v6 (positions 941-1108 of pMeta-1), characterized by high homology (above 90%) between rat and mouse sequences.[118] Random labelling of rA was performed with ^{32}P using T7 Quick Prime Kit (Pharmacia Biotech, Baie d'Urfé, QC, Canada). After washing to a final stringency of 0.5x SSC, 0.1% SDS at 65 C for rA and 42 C for CD44s5 the membranes were exposed to Hyperfilm-MP (Amersham).

Statistical analysis

The chi-square test was applied to analyze contingency tables based on ordinal qualitative variables. Sufficiently large, normally distributed samples of tumor sizes were analyzed using the two-tailed z-test and correlated with ordinal variables using the nonparametric Spearman's correlation coefficient.

RESULTS

DMH-induced neoplastic lesions

All animals injected with DMH developed multiple tumors localized to the mid- and distal colon and rectum. The cecum as well as the proximal colon were invariably free of neoplastic changes. The tumors showed continuous size distribution, with the largest reaching approximately 10 mm diam. The smallest lesions were flat or slightly elevated dysplastic crypt foci. Overall the morphological features were comparable to those reported in the literature.[12 13] The largest lesions consisted of broadly pedunculated epithelial neoplasms usually maintaining a glandular tubular architecture and displaying severe dysplastic changes. Seventeen percent of tumors were invasive and corresponded to carcinomas classified as T1 or T2 in the TNM staging system of human colon cancer.

Although DMH treatment targets mainly the large intestine, other neoplastic lesions were found in experimental animals, with an incidence and histological features corresponding to those already reported.[12 13] Of note the experimental mice had uterine sarcomas and perianal sebaceous neoplasms affecting together approximately 20% of animals. Both the colon tumors as well as the extracolonic lesions were relatively well tolerated and only one animal had to be sacrificed before completion of the protocol. No significant pathology, including dysplastic crypts, was observed in the control group.

CD44 immunostaining

In the normal murine colon only those epithelial cells located in the basal quarter of crypts gave a positive immunoreaction with the KM81 antibody (Fig. 1a). CD44 was also expressed in intramuscular neural ganglia, lymphoid follicles, vessel walls and most stromal elements of lamina propria. This pattern was identical in control animals and in nonneoplastic areas of DMH-treated mice. Dysplastic crypts, found exclusively in DMH-injected group, were characterized by a spectrum of CD44 immunostaining.

One hundred and ten neoplasms (1 to 13 tumors/animal, median = 5) were found in 19 animals and all were graded on degree of differentiation, presence or absence of invasion and immunostaining intensity of neoplastic as well as stromal cells.

Thirty-seven out of the 110 neoplastic lesions (34%) were composed of uniformly immunoreactive neoplastic cells; of those, 26 were uniformly CD44 negative, whereas 11 were uniformly positive, including five with a strong (+++) immunoreaction. Among 73 (66%) tumors composed of variably stained neoplastic cells, one pattern of immunoreactivity tended to predominate. The most commonly found heterogeneous lesions were characterized by scattered areas of CD44 positive neoplastic cells among large areas of negative epithelial cells. The cells of the surface epithelium were often strongly CD44 immunoreactive and not infrequently a sharp transition from CD44 positive to negative superficial epithelium was seen (Fig. 1b). CD44 was overexpressed at an early stage of colorectal carcinogenesis, as demonstrated by the strong immunostaining of dysplastic crypt foci in flat mucosa (Fig. 2a). Some advanced lesions also demonstrated strong and uniform CD44 immunopositivity (Fig. 2b). The results of the immunostaining and morphological analysis are presented in Table 1.

Using the Spearman's rank correlation test, we demonstrated an inverse relationship between the predominant pattern of immunostaining intensity of neoplastic cells and tumor size (rho = -0.26, p value for rho = 0.0066). Larger tumors tended to be mainly CD44 negative, whereas the smaller ones were not infrequently positive. Invasion had a significant association with low CD44 expression of the tumor cells (chi-square test, $p < 0.025$).

Stromal cells of all tumors were invariably CD44 positive, and the majority (70/110) of neoplasms had a level of stromal cell immunostaining comparable to normal colon. In poorly differentiated tumors however, stromal elements were more intensely CD44 immunoreactive compared with those of low-grade neoplasms (chi-square test, $p < 0.05$).

The only other significant correlation was that between sex of animals and tumor grades, female mice developing high grade lesions more frequently than males (chi-square

test, $p < 0.01$). There were no other detectable differences, including CD44 immunoreaction intensity, between tumors induced in mice of either sex.

Expression of CD44 mRNA

Amplification of cDNA using primers designed to detect all CD44 isoforms revealed a complex pattern of products, present both in the normal colon as well as in the tumors. In 15/17 pairs of normal-tumor samples the bands obtained from the colonic tumors were stronger, compared with normal autologous colons. In fact the bands were at the border of detectability in most normal colons. The differences of band intensity were especially pronounced for the slower migrating isoforms, whereas expression of the standard CD44 transcript in tumors remained only slightly elevated. In one pair the heavier bands were of comparable intensity in normal and neoplastic tissue, whereas in another the signal from tumor amplicons was somewhat weaker. Fig. 3a shows representative results of hybridization with the CD44s5 oligonucleotide probe which anneals to the standard part of the molecule. Because s5 is an exon found in all isoforms of CD44, the CD44s5 probe anneals standard and variant CD44 transcripts. The prominent band at 654 bp corresponds to the expected molecular weight of CD44s region amplified by the primers. It is invariably strongly expressed in tumors, autologous non-neoplastic colon and colon samples from H_20-injected mice. At least seven heavier bands ranging from 800 to ~ 1600 bp were detected in all tumor samples. Their pattern was identical in all tumors examined. Corresponding samples of macroscopically normal colon show identical complex array of transcripts, when these were expressed above the detection threshold. The sizes and number of bands detected in the colon of nine control mice did not differ from the non-neoplastic colon sections from DMH-injected animals. They also exhibited some quantitative variability, with two out of nine samples showing an intensity of their bands almost matching that of the neoplastic tissue, and in the remaining seven being much weaker.

The amplicons synthesized from colons of H_20-injected mice either did not hybridize to the variant CD44 probe or gave a barely recognizable signal. In the DMH-injected animals, the pattern of CD44 variant isoforms was similar to that identified by the CD44s probe (Fig. 3b). The tumor samples featured more intense, but qualitatively identical band ladders compared with autologous non-neoplastic colon specimens. The smallest detected band was of ~ 700 bp, the largest of ~ 1650 bp.

Southern blot analysis with the CD44s5 probe disclosed also an unexpectedly small band of ~ 420 bp, clearly visible in Fig. 3a. This strong, often dominant band was consistently present in PCR product from both normal and neoplastic samples. Its molecular weight was far below the expected weight of CD44s amplicon.

In order to explore a possible relationship between CD44 transcript and protein expression the PCR products were compared in two groups of four tumors with positive and negative CD44 immunostaining. No correlation between CD44 immunostaining and RT/PCR results was observed and in particular some tumors with no CD44 immunostaining were characterized by high level of transcripts (data not shown).

DISCUSSION

Data obtained in this study contribute to defining alterations of a cell adhesion molecule that plays an important role in the mechanism of colorectal carcinogenesis. We have demonstrated that the mouse model of colon cancer is characterized by overexpression of a wide range of CD44 mRNA species, coding for both standard and variant isoforms. Additionally, CD44 protein was shown to be upregulated at the very early stages of carcinogenesis, morphologically identified as a single dysplastic crypt. In well defined tumors, the expression of CD44 protein by neoplastic cells was markedly heterogeneous and tended to be weaker in more advanced neoplasms.

Despite the fact that the DMH-induced murine colon adenoma/carcinoma model is widely used in cancer research, many aspects of its pathobiology still remain unclear. The exact mechanisms of DMH carcinogenesis are not exactly known. Metabolite(s) of DMH can cause direct DNA damage via excessive alkylation and creation of single strand breaks.[19,20] The role of mutations of known oncogenes is yet to be investigated, although it is known that the p53 gene is not mutated.[21,22] In this report, we focused on a molecule known to be abnormally expressed in human colon cancer and whose pathology relies on the derangement of alternative exons splicing. Human and mouse CD44 exon sequences are highly conserved, with the intron-exon organization as well as the key HA binding motifs essentially the same in both species.[5,18,23] The only major exception involves exon v1 which in mice is rarely expressed and in humans always spliced out due to the presence of a stop codon.[18] In this study we demonstrated the consistent amplification of a transcript product of ~420 bp, which is over 200 bp shorter than the expected length of the supposedly smallest isoform (CD44s). This amplicon may represent the product of an unusual alternative splicing, whereby one of the regions traditionally presumed to be constantly expressed in all transcripts is excised. We are currently attempting to clone and sequence this amplicon. Cryptic, rarely used splicing sites have been recently reported not only in murine but also in human CD44.[24,25]

Normal human colonic epithelial cells do not express CD44 at the protein level, with the exception of cells located at the base of the crypts.[2] Our results obtained with a pan-CD44 antibody KM81 showed essentially the same pattern of staining in the murine colon. Several reports based on immunoperoxidase methods applied to human surgical specimens present evidence that colon adenoma and carcinoma cells generally overexpress CD44 compared with normal colonic epithelial cells.[2,11,26] Nonetheless these studies show that even in neoplasms, only a minority of cells are positive when stained with pan-CD44 antibodies. The majority of murine tumors investigated in our study demonstrated a similar pattern of CD44 expression, with clusters or zones of CD44 positive cells intermingled with CD44 negative areas.

The significance of CD44 overexpression in colonic neoplasms is a matter of current debate. Some studies have reported lack of association between pan-CD44 immunoreactivity and features such as grade of differentiation, depth of invasion or tumor size.[11 27] In contrast, Jackson *et al* found, in a study evaluating CD44 immunostaining of colon tumors, an inverse relationship between CD44 protein expression and the degree of dysplasia in adenomas[28]; in addition, in their study, the metastatic carcinomas show weaker CD44 expression than the non-metastatic carcinomas. Although in our study the degree of dysplasia was not graded separately, we observed similar trends, linking some other parameters of tumor progression and expression of CD44 in an inverse manner: invading murine tumors exhibited a predominantly negative immunoreaction of neoplastic cells more frequently than the non-invading neoplasms; size of tumors also correlated inversely with the predominant CD44 expression pattern among neoplastic cells. Thus tumor progression in mouse colon neoplasia was associated with weaker overall CD44 expression. These results are consistent with those reported recently in human bladder carcinoma in which overall CD44 immunoreactivity is lost in the more advanced stages.[29] Reports suggest that in advanced human colon cancer, this loss may be selective, involving CD44 isoforms containing products of exon v6.[10 30] Since the repertoire of available mouse CD44 reagents does not yet include antibodies directed against variant sequences, this specific issue could not be addressed in our study.

Some cases of human colon cancer have an undetectable or very weak expression of CD44 proteins on the surface of neoplastic cells at the invasion front.[27] We found that overall CD44 expression was downregulated at the invading front of DMH- induced colon carcinomas. Globally, the human and murine data indicate that invasion is characterized by, and in fact might depend on, the decrease or loss of CD44-mediated adhesion. This is in marked contrast to the cohesive growth of tumor nests proximal to the invading front where CD44 expression is high and possibly necessary for a more static tumor growth.

We also observed CD44 overexpression and heterogeneity in the earliest lesions morphologically identifiable as colon cancer precursors. To the best of our knowledge,

CD44 immunostaining in the dysplastic crypts have not yet been described in human or in animal models. Overexpression of CD44 in ~40% of small human adenomas is reported by Kim *et al.*[26] and occurs before K-ras and p53 mutations. Since we observed it in dysplastic crypt foci, this suggests that it may occur even earlier.

Increased stromal CD44 staining has been previously described in human colon tumors.[27] The present study showed that DMH-induced murine tumors CD44 immunoreactivity was increased in the stromal cells of poorly differentiated neoplasms compared with those of the lamina propria of the normal mucosa. This phenomenon may reflect induction of CD44 synthesis in stromal fibroblasts. The mechanism of this dual - epithelial and stromal - induction is not known.

Abnormal expression of the CD44 gene can be also traced to the transcriptional step using techniques like RT-PCR, northern blot or *in situ* hybridization. Overexpression of variant transcripts, containing products of various alternatively spliced exons distinguishes neoplastic tissue from normal colon.[3 31 32] Recently published *in situ* hybridization studies unequivocally associate CD44v mRNAs with neoplastic cells and exclude stromal elements as a possible source of variant isoforms.[27 33] Although activated lymphocytes are capable of producing CD44v6 [34], DMH-induced tumors are not accompanied by a prominent lymphoid infiltrate, as was found in this study and the literature.[12] Results of our RT-PCR showed a complex array of CD44 isoforms, the majority of them (including v6), with a strong tendency to overexpression in tumor samples compared with normal colon. Based on human data we can reasonably suppose that those numerous isoforms are produced by murine neoplastic epithelial cells and not by fibroblasts or leukocytes. In keeping with normal human colon the normal mouse mucosa displays variant CD44 at low levels.[31 35] The intensity of signal obtained from non-neoplastic samples in our study was generally much weaker, but the pattern of size and the relative abundance of isoforms was the same in the normal colon and the cancer tissue. Some variability between normal samples might reflect an inherent drawback of PCR or, more probably, a real interindividual variation in CD44 expression within the same normal organ, reported also by others.[30] Besides this

important quantitative difference in CD44 variant isoform expression, we detected no tumor- specific bands or bands selectively lost in the tumor tissue. It may be of interest that a sample from a murine uterine sarcoma demonstrated a completely different pattern of CD44 isoforms, with different molecular weights and relative arrangement of bands (not shown).

RT-PCR performed with four tumors showing strong CD44 immunoperoxidase staining and four tumors characterized by completely negative immunostaining revealed lack of any obvious correlation between variant transcript abundance and immunostaining intensity. This fact, together with the presence of a whole ladder of CD44v isoforms in normal samples adds support to the notion that there is a major dysfunction of CD44 splicing in colon carcinoma. Many CD44 transcripts are not used for protein synthesis in colon cancer cells.[36] The alteration of splicing machinery can also result in retention of introns in colon cancer.[37] It is conceivable that the overexpression of numerous species of variant isoforms of CD44 transcripts results also in structurally different CD44 protein isoforms with altered adhesion functions.

Acknowledgments

Supported by a Hans and Eugenia Jütting Fellowship to Z.R. and an operating grant from the Medical Research Council of Canada (MT-10315).

TABLE 1: CD44 protein expression and other features of colon tumors induced by DMH injections in ICR/Ha mice

CD44 immuno-staining	neoplastic cells, predominant pattern	tumors	(-) 74 [67.3]	(+) 13 [11.8]	(++) 5 [4.5]	(+++) 18 [16.4]	
	neoplastic cells, secondary pattern		homoge-neous 37 [33.6]	(-) 12 [11]	(+) 25 [22.7]	(++) 17 [15.5]	(+++) 19 [17.3]
	stromal cells		(+) 25 [22.7]	(++) 70 [63.6]	(+++) 15 [13.6]		
tumor size [mm]			min=0.4 max=10.0 avg=2.8 sd=1.82				
histological grade			gr 1 16 [14.5]	gr 2 69 [62.7]	gr 3 25 [22.7]		
evidence of invasion			no invasion 95 [86.4]		invading tumors 15 [13.6]		
sex			66 [60] tumors in 11 males		44 [40] tumors in 8 females		

Spearman's rho=-.26 p<0.01

chi² p<0.025

chi² p<0.05

chi² p<0.01

One hundred ten tumors were immunostained with a pan-CD44 monoclonal antibody (KM81). Scoring of immunoreaction and tumor grading are detailed in Materials and Methods. Number of tumors given for each category (except "tumor size"). Percentage values in brackets. The tumors were graded according to their degree of differentiation.[16]

FIGURE LEGENDS

Fig.1. Patterns of CD44 expression in frozen sections of mouse colon detected with KM81 monoclonal antibody. (a) Normal mucosa with CD44 positivity confined to basal epithelial cells. Magnification, x 125. (b) DMH-induced adenocarcinoma. CD44 positive neoplastic cells predominate within the superficial zone of the tumor. Some of them cluster also near the invasion front. Magnification, x 90. Immunoperoxidase, light hematoxylin counterstaining.

Fig.2. Overexpression of CD44 detected with KM81 monoclonal antibody in murine colon at different stages of DMH-induced carcinogenesis. (a) Horizontally oriented dysplastic gland with strong CD44 expression, located in upper zone of mucosa. Note also the small discrete group of abnormal epithelial cells characterized by much weaker immunostaining at the left side of the crypt. Magnification, x 220. (b) Adenocarcinoma characterized by uniform overexpression of CD44 protein. CD44 immunostaining of malignant glands is most prominent within basal and parabasal cytoplasm of neoplastic cells. Magnification, x 550. Immunoperoxidase, light hematoxylin counterstaining.

Fig 3. CD44 isoforms detected by RT-PCR/Southern blot analysis in colon tumors (T) and corresponding autologous normal samples (N) from DMH-injected mice. (a) Hybridization with probe CD44s5 detecting all CD44 isoforms. An amplicon of 654 bp corresponding to the standard form of CD44 (CD44s) (*arrow*) and an unexpectedly low weight (380 bp) PCR product (*arrowhead*) are prominent in both normal and tumor samples. In 90% (17/19) of cases the heavier CD44 isoforms were overexpressed in neoplastic samples, compared with autologous normal colon. (b) Hybridization with probe rA specific for CD44 exons v5-v6, showing overexpression of selected variant isoforms in the neoplastic tissue. The arrow and the arrowhead correspond to ~950 and ~700 bp respectively.

Fig. 1a.

Fig. 1b.

Fig. 2a.

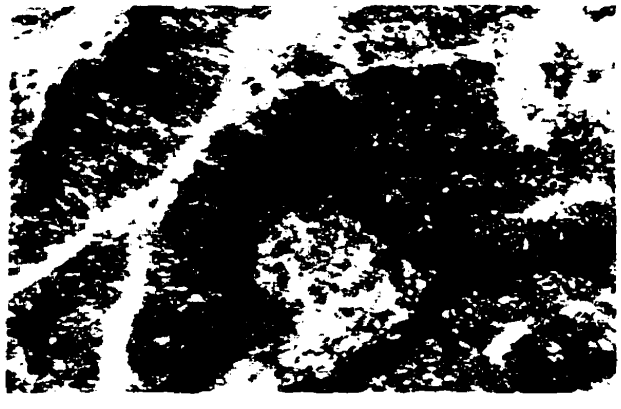


Fig. 2b.

Fig. 3a.

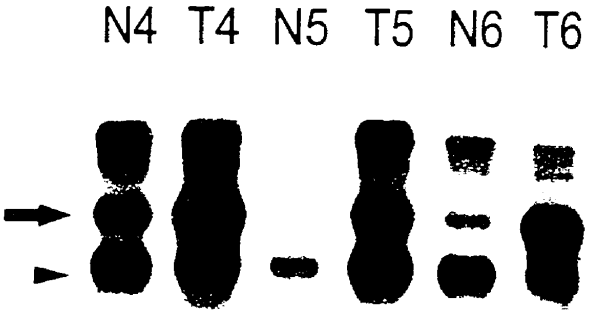


Fig. 3b.

REFERENCES

1 Günthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, et al. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991;**65:**13-24.

2 Heider KH, Hofmann M, Hors E, van den Berg F, Ponta H, Herrlich P, Pals ST. A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J Cell Biol* 1993;**120:**227-33.

3 Matsumura Y, Tarin D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 1992;**340:**1053-8.

4 Rudzki Z, Jothy S. CD44 and the adhesion of neoplastic cells. *J Clin Pathol:Mol Pathol* 1997 (in press).

5 Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* 1992;**89:**12160-4.

6 Yang B, Yang BL, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J* 1994;**13:**286-96.

7 Bourguignon LY, Walker G, Suchard SJ, Balazovich K. A lymphoma plasma membrane-associated protein with ankyrin-like properties. *J Cell Biol* 1986;**102:**2115-24.

8 Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 1994;**126:**391-401.

9 Mulder JW, Wielenga VJ, Polak MM, van den Berg FM, Adolf GR, Herrlich P, et al. Expression of mutant p53 protein and CD44 variant proteins in colorectal tumorigenesis. *Gut* 1995;**36:**76-80.

10 Finke LH, Terpe HJ, Zorb C, Haensch W, Schlag PM. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1995;**345:**583.

11 Ichikawa W. Positive relationship between expression of CD44 and hepatic metastases in colorectal cancer. *Pathobiology* 1994;**62:**172-9.

12 Haase P, Cowen DM, Knowles JC, Cooper EH. Evaluation of dimethylhydrazine induced tumours in mice as a model system for colorectal cancer. *Br J Cancer* 1973;**28:**530-43.

13 Toth B, Malick L, Shimizu H. Production of intestinal and other tumors by 1, 2-dimethylhydrazine dihydrochloride in mice. I. A light and transmission electron microscopic study of colonic neoplasms. *Am J Pathol* 1976;**84:**69-86.

14 Moen CJ, Groot PC, Hart AA, Snoek M, Demant P. Fine mapping of colon tumor susceptibility (Scc) genes in the mouse, different from the genes known to be somatically mutated in colon cancer. *Proc Natl Acad Sci USA* 1996;**93:**1082-6.

15 Miyake K, Medina KL, Hayashi S, Ono S, Hamaoka T, Kincade PW. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J Exp Med* 1990;**171:**477-88.

16 Lewin KJ, Riddel RH, Weinstein WM: Gastrointestinal Pathology and Its Clinical Implications. New York, Igaku-Shoin Medical Publishers, 1992, pp 1273-1275.

17 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162:**156-9.

18 Screaton GR, Bell MV, Bell JI, Jackson DG. The identification of a new alternative exon with highly restricted tissue expression in transcripts encoding the mouse Pgp-1 (CD44) homing receptor. Comparison of all 10 variable exons between mouse, human, and rat. *J Biol Chem* 1993;**268:**12235-8.

19 Boffa LC, Gruss RJ, Allfrey VG. Aberrant and nonrandom methylation of chromosomal DNA-binding proteins of colonic epithelial cells by 1,2-dimethylhydrazine. *Cancer Res* 1982;**42:**382-8.

20 Bolognesi C, Mariani MR, Boffa LC. Target tissue DNA damage in inbred mouse strains with different susceptibility to the colon carcinogen 1,2-dimethylhydrazine. *Carcinogenesis* 1988;**9:**1347-50.

21 Okamoto M, Ohtsu H, Kominami R, Yonekawa H. Mutational and LOH analyses of p53 alleles in colon tumors induced by 1,2-dimethylhydrazine in F1 hybrid mice. *Carcinogenesis* 1995;**16:**2659-66.

22 Okamoto M, Ohtsu H, Miyaki M, Yonekawa H. No allelic loss at the p53 locus in 1,2-dimethylhydrazine-induced mouse colon tumours: PCR-SSCP analysis with sequence-tagged microsatellite site primers. *Carcinogenesis* 1993;**14:**1483-6.

23 Tölg C, Hofmann M, Herrlich P, Ponta H. Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res* 1993;**21:**1225-9.

24 Ermak G, Jennings T, Robinson L, Ross JS, Figge J. Restricted patterns of CD44 variant exon expression in human papillary thyroid carcinoma. *Clin Cancer Res* 1996;**56:**1037-42.

25 Yu Q, Toole BP. A new alternatively spliced exon between v9 and v10 provides a molecular basis for synthesis of soluble CD44. *J Biol Chem* 1996;**271:**20603-7.

26 Kim H, Yang XL, Rosada C, Hamilton SR, August JT. CD44 expression in colorectal adenomas is an early event occurring prior to K-ras and p53 gene mutation. *Arch Biochem Biophys* 1994;**310:**504-7.

27 Gorham H, Sugino T, Woodman AC, Tarin D. Cellular distribution of CD44 gene transcripts in colorectal carcinomas and in normal colonic mucosa. *J Clin Pathol* 1996;**49:**482-8.

28 Jackson PA, Green MA, Pouli A, Hubbard R, Marks CG, Cook MG. Relation between stage, grade, proliferation, and expression of p53 and CD44 in adenomas and carcinomas of the colorectum. *J Clin Pathol* 1995;**48:**1098-101.

29 Sugino T, Gorham H, Yoshida K, Bolodeoku J, Nargund V, Cranston D, et al. Progressive loss of CD44 gene expression in invasive bladder cancer. *Am J Pathol* 1996;**149:**873-82.

30 Gotley DC, Fawcett J, Walsh MD, Reeder JA, Simmons DL, Antalis TM. Alternatively spliced variants of the cell adhesion molecule CD44 and tumour progression in colorectal cancer. *Br J Cancer* 1996;**74:**342-51.

31 Wielenga VJ, Heider KH, Offerhaus GJ, Adolf GR, van den Berg FM, Ponta H, et al. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. *Cancer Res* 1993;**53:**4754-6.

32 Imazeki F, Yokosuka O, Yamaguchi T, Ohto M, Isono K, Omata M. Expression of variant CD44-messenger RNA in colorectal adenocarcinomas and adenomatous polyps in humans. *Gastroenterology* 1996;**110:**362-8.

33 Gorham H, Sugino T, Bolodeoku J, Yoshida K, Goodison S, Tarin, D. Distribution of CD44 messenger RNA in archival paraffin wax embedded tumours and normal tissues viewed by in situ hybridisation. *J Clin Pathol:Mol Pathol* 1996;**49:**M147-M150.

34 Koopman G, Heider KH, Horst E, Adolf GR, van den Berg F, Ponta H, et al. Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med* 1993;**177**:897-904.

35 Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Günthert U. Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol* 1994;**124**:71-82.

36 Woodman AC, Sugiyama M, Yoshida K, Sugino T, Borgya A, Goodison S, et al. Analysis of anomalous CD44 gene expression in human breast, bladder, and colon cancer and correlation of observed mRNA and protein isoforms. *Am J Pathol* 1996;**149**:1519-30.

37 Yoshida K, Bolodeoku J, Sugino T, Goodison S, Matsumura Y, Warren BF, et al. Abnormal retention of intron 9 in CD44 gene transcripts in human gastrointestinal tumors. *Cancer Res* 1995;**55**:4273-7.

FINAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

The research summarized in the current dissertation provided original data on the expression of CD44 protein and mRNA in a murine model of colon cancer. It proved that CD44 protein may be overexpressed at the earliest stages of colon carcinogenesis, when a lesion presents morphologically as a dysplastic crypt. In overtly neoplastic lesions, analogous to human adenomas and carcinomas, CD44 positive cells usually formed randomly distributed clusters. This contrasted sharply with the strictly regulated pattern of distribution of CD44 positive cells found in the normal colon, where the immunoreactivity was restricted to the proliferative zone at the basal parts of crypts. In a murine model, similarly to its human counterpart, there is a tendency towards downregulation of CD44 protein in more advanced tumors. CD44 mRNAs are almost invariably overexpressed in colon tumors as opposed to normal colon tissue. However, this overexpression is not accompanied by creation of new isoforms in the murine model. Taken together, the neoplastic transformation of colonic epithelial cells is associated with a global upregulation of CD44 isoforms at the transcriptional stage. This is accompanied by a more haphazard disorganization of mRNA translation resulting in strong heterogeneity of neoplastic and preneoplastic lesions in terms of CD44 protein expression. It seems that CD44 overexpression is a characteristic but not indispensable element of a colon carcinogenesis at all its stages.

Finally, application of RT-PCR/Southern blot with a probe specific to the second exon of murine CD44 revealed an unexpectedly small band of approximately 380 bp, invariably and abundantly present both in normal colon and tumor samples. This potential amplicon is ~200 bp smaller than the smallest (standard) form of CD44 so far described and the work aimed at elucidation of its nature is currently in progress. It may be a result of a new splicing site within CD44 pre-mRNA, a product of a distinct but CD44 related gene or even a peculiar PCR artefact. Possible research strategies involve direct sequencing of resolved and purified amplicon or cloning it into a plasmid with subsequent sequence

analysis. If it represents a new extremely small isoform of CD44, it is possible that it lacks one or both hyaluronan binding motifs, having radically different properties in terms of regulation of cell adhesion. Solving this riddle would shed some new light into molecular biology of this intriguing protein.

The research outlined in the present thesis is an important first step in the characterization of CD44 expression in the murine colon cancer model. Abnormalities of mRNA production can also be investigated using techniques combining molecular biology with morphology, like *in situ* hybridization or *in situ* PCR. Recent successful application of *in situ* hybridization to human CD44 has been outlined in the review article (Rudzki and Jothy 1997). Precise identification of CD44 isoforms produced by various cells, including the stromal ones, by exon-specific probes could be accompanied by immunostaining of the same or serial sections. This would probably allow a solution to the puzzling issue of frequent discrepancies between expression of CD44 at the protein and mRNA levels.

It is still unclear if CD44 glycoprotein found in abundance in some murine colon tumors contributes to any special biological properties of the neoplastic cells, or is associated with other molecular abnormalities defining the neoplastic phenotype. This issue could be addressed in further studies in several ways. For example, CD44 positivity assessed by immunostaining could be correlated with accepted parameters of cellular proliferative activity such as Ki-67 or PCNA expression. An attractive possibility includes functional experiments, whereby living cells harvested from murine tumors could be sorted by using flow cytometry. Resulting subpopulations differing in CD44 positivity could be tested for those properties typically associated with the malignant phenotype like adhesion to extracellular matrix, aggregation, migration, or *in vitro* invasion in a short term culture. These tests do not require the use of long term cultures of cell lines. Attempts to establish such cell lines, differing in CD44 expression would be challenging but could result in the creation of a valuable research tool. A cell sorter would also allow an investigation of discrete tumor cell populations using RT-PCR/Southern blot and Western blot.

Overall it is relevant to ask in the future which and how specific isoforms of CD44 modulate adhesion and antiadhesion in tumor cells during migration and invasion. At a more basic level, it is also important to understand the cellular mechanisms of CD44-dependant adhesion, investigating the possible interactions between CD44 and cytoplasmic molecules or components of the cell membrane.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor, Dr. Serge Jothy thanks to whom I started my adventure with molecular biology. For two years not only did he provide me with research facility but also with constant and invaluable inspiration and support.

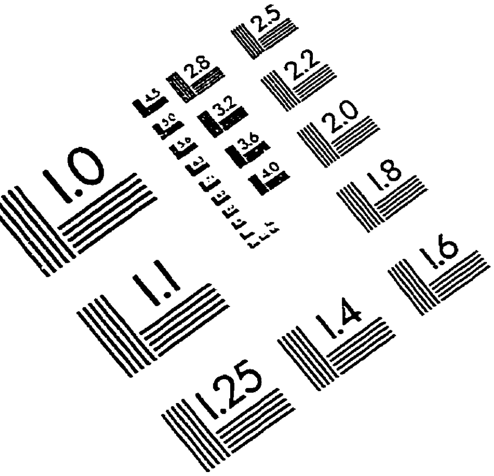
I am deeply indebted to Mr. Lam LeDuy, working as a technician in Dr. Jothy's lab. He devoted a lot of his precious time and patience to teach me both practical as well as theoretical aspects of molecular biology, sharing with me his extraordinary knowledge and skills.

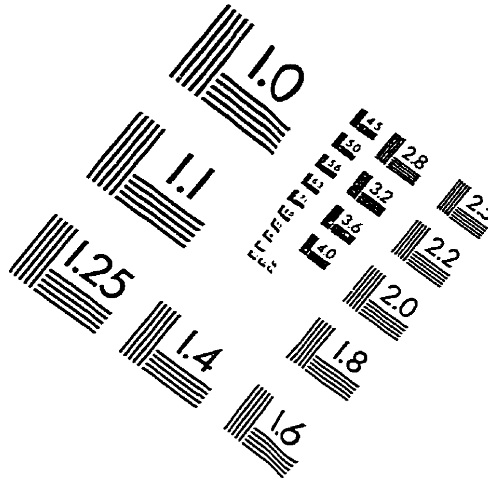
The generosity of Donors of Hans and Eugenia Jütting Fellowship made my studies at MCGill possible, providing me with financial resources sufficient to cover costs of travel, tuition fees and living expenses.

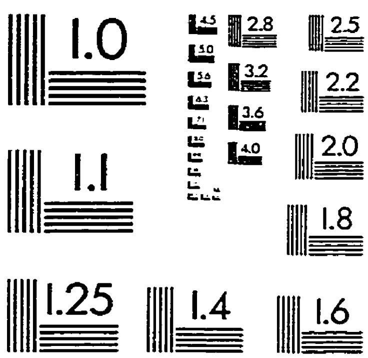
I would like to thank Dr. René P. Michel and Dr. Orest W. Blaschuk, who agreed to be my research coadvisors and Dr. Gerald Prud'homme, whose equipment I have used. Additionally, Dr. Michel reviewed the manuscript of the present thesis, providing numerous valuable suggestions. Graduate students from the Department of Pathology - Andrea Herrera-Gayol, Lijin Liang, Ciro Piccirillo and Keli Song deserve acknowledgements for sincere friendship.

Finally, I can not forget to thank my wife Natalia for her patience and understanding which she exhibited almost constantly, even when I was spending most of my time in the lab.

IMAGE EVALUATION TEST TARGET (QA-3)

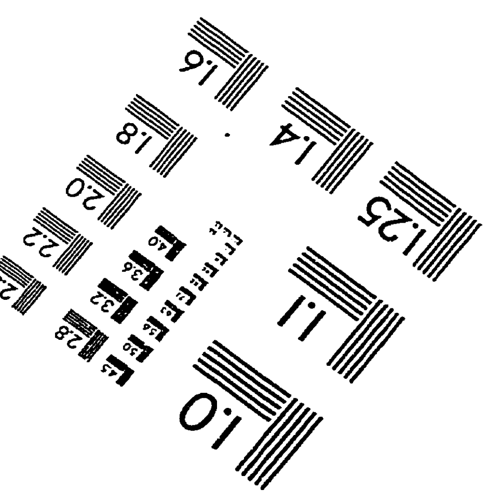


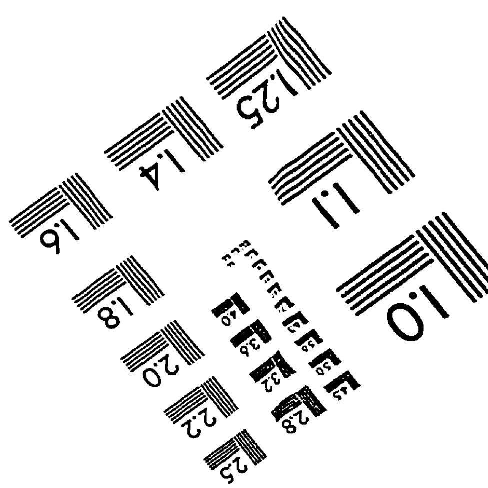




150mm

6″





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989