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ORIGINAL PAPERS

INTERSTITIAL CAJAL-LIKE CELLS AND BILE LITHOGENICITY IN THE PATHOGENESIS OF GALL-STONE DISEASE

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Gall-stone disease constitutes a serious clinical problem and is the most frequent cause of elective cholecystectomies. There are many etiopatogenic factors however; lithogenic bile and its stasis due to gall-bladder hypomotility seem to be the most important. In recent years discovery of pacemaker function of Interstitial Cells of Cajal changed our understanding of smooth muscle physiology and helped to disclose many gastrointestinal motility disorders.

The aim of the study was identification and quantification of interstitial Cajal-like cells (ICLCs) in gall-bladder muscle wall from patients with cholelithiasis and in gall-stone-free controls, as well as determination of the relationship between the number of ICLCs and Cholesterol Saturation Index (CSI) of bile in both analyzed groups.

Material and methods. 20 patients operated for symptomatic cholelithiasis were enrolled into the study group. The control group consisted of 20 patients operated for pancreatic head tumors, with no pre- and intraoperative signs of gall-stones. Identification of ICLCs in the gall-bladder was performed by means of double immunofluorescence technique with anti c-Kit and anti-mast cell tryptase antibodies. Quantitative analysis was carried out under fluorescence microscopy conjoined with image analysis software. Bile samples were used for calculation of CSI.

Results. ICLCs were detected within gall-bladder muscle wall. Number of ICLCs was statistically significantly lower in patients from the study group as compared to control. The study also revealed statistically significantly higher CSI in the study group.

Conclusions. The quantity of ICLCs is diminished in the gall-bladder from patients with cholelithiasis and there is negative correlation between the number of ICLCs and CSI of bile. Regarding the role of ICCs in regulation of GI tract motility, it appears that reduction in their number may be important etiopatogenic factor of cholelithiasis.

Key words: bile lithogenecity, interstitial Cajal-like cells, gall-stone disease, c-Kit, mast cells

Cholelithiasis is a common disorder in the developed countries. Surgical procedures of the gall-bladder due to cholelithiasis are the most common surgical operations at the departments of general surgery. Several factors are involved in the pathogenesis of this disease, but bile stasis related to impaired motility of the gall-bladder and lipid contents of the bile play a dominant role here (1-4). Cholesterol deposits are the most common among

all concrements found in the biliary system (2, 5). Recently our understanding of physiology of the gastrointestinal smooth muscle system has changed due to studies of the population of the interstitial cells of Cajal. These cells were described for the first time by a Spanish neuroanatomist Santiago Ramony Cajal (1852 -1934) in 1889; they were identified in the intestinal wall using non-specific methods of impregnation with silver salts and methylene blue staining in tissues of a living organism (6, 7). However, only discovery of a reaction of the interstitial cells with an antibody against c-Kit antigen opened a new era in the methodology of identification of these cells (8).

Interstitial cells of Cajal (ICC) exhibit membrane expression of c-Kit receptor. c-Kit protein (CD117), with a molecular weight of 145 kDa, is encoded by a protooncogene c-kit located in the locus W (White Spotting), in humans on a long arm of chromosome 4, close to genes encoding e.g. growth factors (9). C-Kit belongs to transmembrane receptors and its domains, of tyrosine kinase type (RTKs – Receptor Tyrosine Kinases) that are activated by growth factors, are involved in the signal transduction from the external cellular environment to its inside. This process results in modification of gene expression and biosynthesis of specific proteins. Thus c-Kit is involved in the regulation of cellular survival, proliferation and differentiation. The interstitial cells of Cajal can be also found in multiple organs other than intestine where they are called Cajal-like cells and recently they are also referred to as telocytes, but their morphology and function were best understood in the gastrointestinal system. So far we know that these cells play a role of pacemaker cells and their dysfunction can underlie gastrointestinal motility disorders (10). The role of ICC involves generation and modulation and modulation of slow waves in the gastrointestinal system, their propagation and coordination and mediation of neurotransmission between autonomic nervous system and muscle cells.

The aim of the study was to identify and estimate the number and distribution of Cajal-like cells in the gall-bladder wall in patients with cholelithiasis and in non-cholelithiatic, control patients. Furthermore, secondary aims included determination of correlation between the number of Cajal-like cells and bile cholesterol saturation index and lipid composition of the bile in both study groups.

MATERIAL AND METHODS

The study enrolled 40 patients aged 27 to 80 years who underwent surgical treatment at the 1st Clinic of General, Oncological and Gastroenterological Surgery, Jagiellonian University, Collegium Medicum in Cracow, in 2010. The study group included 20 patients who underwent surgical treatment due to symptomatic cholecystolithiasis. All patients had normal serum bilirubin level before the procedure. Cholecystectomies were performed in an elective setting, using a laparoscopic method. The control group included 20 patients who underwent an elective surgical procedure due to a tumor of the head of the pancreas in whom neither preoperative imaging procedures (ultrasound imaging) nor intraoperative examination revealed cholecystolithiasis. Inclusion criterion for the control group also included normal serum bilirubin level before the surgical procedure. If the tumor was resectable, the patients underwent Whipple's or Traverso – Longmire's pancreatoduodenectomy. If the lesions were unresectable, a gastrointestinal bypass was performed with Braun's intestinal anastomosis. In each of these cases the gall-bladder was removed and subsequently was used for histological testing. Furthermore, in both patient groups during the surgical procedure 2 ml of bile were collected from the gall-bladder to perform biochemical tests.

Gall-bladders underwent gross examination and then were fixed in 4% buffered aqueous solution of paraformaldehyde. A continuous specimen (band) was collected from each gall-bladder and was subsequently embedded in paraffin so as to enable its cutting into preparations having all layers of the gall-bladder wall. The preparations were deparaffinized and hydrated to perform further histological staining.

Interstitial Cajal-like cells were identified on transverse sections of the gall-bladder wall by indirect double immunofluorescence using antibodies against c-Kit receptor and mast cell tryptase to provide better differentiation between Cajal-like cells and mast cells. Number and distribution of interstitial Cajal-like cells in the gall-bladder were determined using a fluorescence microscope Jenamed 2 – Fluores-

cence (Carl Zeiss, Jena, Germany) conjugated with a computer system of image analysis (ProgRes Capture Pro v.2.8, camera CCD ProgRes C12^{Plus}(Jenoptik)).

Samples of the bile collected intraoperatively from the gall-bladder were used in the quantitative analysis of its lipid contents, i.e. to determine the concentrations of: cholesterol, phospholipids and bile acids. Cholesterol and phospholipids were determined using an enzymatic method, while individual bile acids were determined using a HPLC (High Performance Liquid Chromatography) method following their previous extraction. Basing on concentrations of individual lipids, a lithogenicity index (Cholesterol Saturation Index, CSI) was calculated for any bile sample using Carey's charts (11).

Obtained results were presented as arithmetic means, standard deviations and in certain cases, 95% confidence intervals (95% CI). Differences between the study groups were analyzed using non-parametric tests (Mann-Whitney's test). The strength of relationship between features was tested using Pearson's correlation coefficient. $p < 0.05$ was considered significant. Calculations were done using PRISM 5.0 software.

The study was approved by Bioethics Committee of Jagiellonian University (no. KBET/30/B/2010 dated 25 March 2010), in compliance with Declaration of Helsinki.

RESULTS

Immunohistochemical methods (indirect double immunofluorescence staining) identified cells positive for reaction with anti-c-Kit in preparations of human gall-bladder. These cells included both Cajal-like cells and numerous mast cells (mastocytes) that also expressed c-Kit receptor (12). Antibodies against mast cell tryptase that is present in mast cells but absent in Cajal-like cells, enabled differentiation between interstitial Cajal-like cells and mast cells. Therefore, the following were observed in microscopic preparations: c-Kit immunopositive cells (staining red) and tryptase-immunopositive cells (staining green) and furthermore blue stained nuclei which were visualized using appropriate filters. In the quantitative analysis, criteria of Cajal-like cells included presence of the following structures: c-Kit immunopositive, concomitantly tryptase-immunonegative and having a nucleus (to eliminate accidental non-cellular structures from the analysis).

Interstitial Cajal-like cells could be seen throughout the band comprising the gall-bladder fundus, body and neck. Cajal-like cells were identified almost exclusively in the muscular membrane of the gall-bladder where they were oriented parallel to the smooth muscle fibers. Microscopic analysis of the preparations revealed the following distribution of Cajal-like cells: intramuscular ICLC (ICLC-IM), i.e. located between the smooth muscle fibers comprising a single bundle, as well as interbundle ICLC (ICLC-IB), i.e. located in the connective tissue that separated the smooth muscle bundles. Cajal-like cells were usually found as single cells, sometimes in small groups of 2-3 cells, but complex networks of these cells were not found.

Interstitial Cajal-like cells were characterized by their elongated shape, their length was 40 to 60 μm, and sporadic processes could be seen in few preparations. Furthermore few c-Kit immunopositive (concomitantly tryptase-immunonegative) cells, with an oval-like shape were found in the preparations and due to configuration of immune markers were also considered Cajal-like cells (fig. 1).

On the other hand, c-Kit immunopositive mast cells observed in the preparations had usually a round or oval shape and a round, centrally located nucleus, which additionally made their differentiation from Cajal-like cells easier.

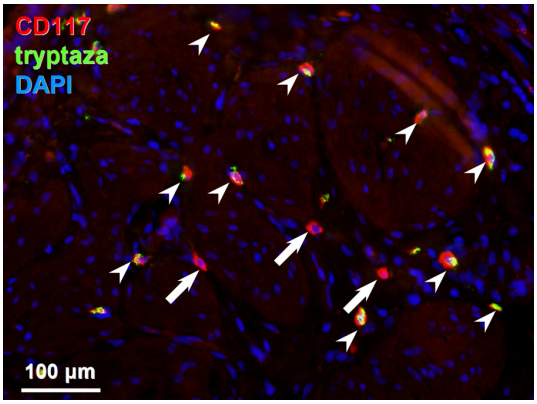

Fig. 1. A gall-bladder, muscular priopria. CD117-immunopositive and tryptase-immunonegative Cajal-like cells (stained red, arrows). CD117-immunopositive and tryptase-immunopositive mast cells (yellow, arrowheads). Nuclei are stained blue with DAPI

An average number of interstitial Cajal-like cells analyzed in 10 consecutive areas of view (AOV) covering fragments of the fundus and body of the gall-bladder in the band collected from the gall-bladder wall in the group of patients with cholelithiasis was 33.5 ± 12.4 and was two-fold lower than in the control group: 70.6 ± 18.2. This difference was statistically significant ($p < 0.001$)

A significantly higher cholesterol saturation index was also found in the study group (with cholelithiasis) versus the control group ($p<0.03$). Furthermore, statistical analysis demonstrated a strong negative correlation between CSI and number of Cajal-like cells in the gall-bladder wall ($r = -0.60$, $p = 0.01$). Table 2 presents average values of cholesterol saturation index in both analyzed patient groups.

Statistically significantly lower concentration of glycocholic acid (GCA) and taurocholic acid (TCA) was found in the gall-bladder bile in the study group versus the control group (average GCA concentration GCA: 35.66 ± 16.66 mmol/l vs 43.44 ± 32.40 mmol/l, average TCA concentration: 9.00 ± 7.66 vs 15.18 ± 11.09 mmol/l). Number of Cajal-like cells exhibited positive correlation with concentration of glycocholic ($r = 0.50$, $p = 0.04$) and taurocholic ($r = 0.32$, $p = 0.05$) acid.

DISCUSSION

Interstitial Cajal-like cells in the bile ducts were identified relatively recently. Their presence in the human gall-bladder was mentioned for the first time in 2000 by Ortiz-Hidalgo et al. who presented a description of stromal tumor of the gall-bladder comprising cells of ICLC phenotype (13). Presence of interstitial Cajal-like cells in the human gall-bladder resected for non-cancer reasons was reported as late as in 2007 by Hinescu E.M. et al. (14) and in 2009 by Ahmadi O. et al. (15). However, currently available literature does not include other reports of the number and distribution of interstitial Cajal-like cells in individual layers of the human gall-bladder.

In this study we found a statistically significant reduction of interstitial Cajal-like cells in the study group (with cholelithiasis) versus the control group (without cholelithiasis). Two-fold reduction of the number of interstitial Cajal-like cells may significantly affect motility of the gall-bladder and result in cholelithiasis. Literature reports existence of correlation between reduced number of ICC with gastrointestinal diseases and abnormal gastrointestinal motility (16, 17, 18). Numerous papers indicate presence of abnormalities of the gall-bladder emptying in patients with cholecystolithiasis (19, 20, 21). On this basis one can hypothesize that abnormal gall-bladder motility may result from low number of Cajal-like cells in the gall-bladder wall and lead to abnormalities in bile outflow, ultimately with formation of concrements in its lumen. The presented results confirm our previous findings that the number of c-Kit immunopositive (concomitantly tryptase-immunonegative) cells that from the phenotypic point of view could correspond to Cajal-like cells, is reduced in patients with cholelithiasis (22).

Some authors claim that impaired gall-bladder motility is evoked by absorption of cholesterol from lithogenic bile through the gall-bladder wall (23). Excessive amounts of cholesterol in the smooth muscles of the gall-bladder wall may stiffen the sarcolemmal membrane and impair the signal transduction mediated by protein G, resulting from CCK-A binding to its receptor, ultimately paralyzing the gall-bladder contractility (24). It remains unknown whether this phenomenon occurs only and exclusively in the smooth muscle cells or also in the interstitial Cajal-like cells. Hu et al. demonstrated in an animal model that high cholesterol diet significantly reduces c-Kit protein as well as c-kit mRNA in the gall-bladder wall in the guinea pig (25).

Table 1. Number of interstitial Cajal-like cells per area of view and mm^2 of tissue

	Study group			Control group		
	average value	SD	95% CI	average value	SD	95% CI
Number of ICLCs per 10 AOVs	33,5	12,4	28,5 – 38,5	70,6	18,2	62,0 – 74,0
Number of ICLCs / mm^2 of tissue	22,67	12,17	21,06 – 28,48	54,62	13,42	47,87 – 61,60

Table 2. Cholesterol saturation index (CSI) of the gall-bladder bile in the study group (n = 20) and in the control group (n = 20)

	Study group		Control group	
	average value	SD	average value	SD
CSI	1,23	0,84	0,78	0,33

This study demonstrated a statistically significantly higher cholesterol saturation index in the study group versus the control group. This means that bile in the study group (with cholelithiasis) was highly lithogenic which was compatible with reports by Admirand and Vlahcevic as well as results by Shoda et al. (26, 27, 28). A statistically significantly lower concentration of glycocholic and taurocholic acid in the gall-bladder bile was found in the study group versus the control group. Lower relative contents of the above mentioned acids may have resulted in higher cholesterol saturation index in the study group. However, on the other hand currently we know that relative oversaturation of bile with cholesterol due to reduction of relative contents of bile salts and/or phospholipids does not have to result in formation of cholesterol stones. This may only occur when additional circumstances occur, such as biliary stasis associated with reduced number of Cajal-like cells in the gall-bladder wall (29). Moreover, bile oversaturated with cholesterol may be periodically found in healthy humans who do not have evidence of cholelithiasis (30, 31). Therefore, irrespective of lipid contents or cholesterol saturation index, additional factors that affect the risk of cholelithiasis must be always taken into account, in particular in patients with relatively low index indicating low bile saturation with cholesterol. Therefore in this study we correlated cholesterol saturation index with the number of Cajal-like cells in both patient groups. Statistically significantly higher cholesterol saturation index was found in the study group versus the control group and an average number of Cajal-like cells per area of view was statistically significantly lower in the study group versus the control group. In view of role that the interstitial Cajal-like cells play in the gastrointestinal motility, therefore in the gall-bladder motility too, such correlation should be considered relevant for the etiopathogenesis of cholelithiasis.

A statistically significant positive correlation was found between an average number of Cajal-like cells and concentration of glycocholic and taurocholic acid in the study group. However, no correlation was found between CSI and GCA and TCA in the presented group. This means that reduced number of Cajal-like cells, and thus impaired gall-bladder motility, may be the cause of increased bile lithogenicity.

In summary, number of Cajal-like cells in the gall-bladder wall in patients with cholelithiasis is reduced and exhibits negative correlation with cholesterol saturation index. In view of the role of interstitial cells in the regulation of the gastrointestinal motility, one can claim that reduction of their number may be an important factor in the pathogenesis of cholelithiasis. Basing on obtained results, it seems warranted to modify current concept of pathogenesis of cholecystolithiasis in which main role is ascribed to bile lithogenicity, adding abnormal gall-bladder motility related to reduced number of interstitial Cajal-like cells in the gall-bladder wall.

CONCLUSIONS

1. Interstitial Cajal-like cells are present in the gall-bladder wall in humans (in its fundus, body and neck).
2. Double immunofluorescence using c-Kit antigen and a specific marker of mast cells – mast cell tryptase is a reliable method of determination of interstitial Cajal-like cells and allows for their differentiation from numerous mast cells that are present in the tissue specimens of the gall-bladder.
3. Interstitial Cajal-like cells in the gall-bladder are located predominantly in the muscular membrane.
4. Number of interstitial Cajal-like cells in the gall-bladder is reduced in patients with cholecystolithiasis.
5. Number of interstitial Cajal-like cells correlates negatively with cholesterol saturation index.
6. Two-fold reduction of interstitial Cajal-like cells in the gall-bladder wall may result in its abnormal motility, which, together with increased cholesterol saturation index, may result in cholelithiasis.

REFERENCES

1. *Apstein MD, Carey MC*: Pathogenesis of cholesterol gallstones: a parsimonious hypothesis. *Eur J Clin Invest* 1996; 26: 343-52.
2. *Paumgartner G, Sauerbruch T*: Gallstones: pathogenesis. *Lancet* 1991; 338: 1117-21.
3. *Portincasa P, Moschetta A, Palasciano G*: Cholesterol gallstone disease. *Lancet* 2006; 368: 230-39.
4. *Strasberg SM*: The pathogenesis of cholesterol gallstones a review. *J Gastrointest Surg* 1998; 2: 109-25.
5. *Trotman BW*: Pigment gallstone disease. *Gastroenterol Clin North Am* 1991; 20: 111-26.
6. *Cajal SR*: Nuevas aplicaciones del metodo de coloracion de Golgi. *Gaceta Medica Catalana* 1889; 12: 613-16.
7. *Cajal SR*: Sur le ganglions et plexus nerveux de l'intestin. *C R Soc Biol Paris* 1893; 5: 217-23.
8. *Torihashi S, Ward SM, Nishikawa S* et al.: *C-kit*-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell Tissue Res* 1995; 280(1): 97-111.
9. *Huang E, Nocka K, Beier D* et al.: The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* 1990; 63: 225-233.
10. *Huizinga JD, Zarate N, Farrugia G:* Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. *Gastroenterology* 2009; 137(5): 1548-56.
11. *Carey MC:* Critical tables for calculating the cholesterol saturation of native bile. *J Lipid Research* 1978; 19: 945-55.
12. *Rumessen JJ, Vanderwinden JM*: Interstitial Cells in the Musculature of the Gastrointestinal Tract: Cajal and Beyond. *International Review of Cytology* 2003; 229: 115-208.
13. *Ortiz-Hidalgo C, de Leon Bojorge B, Albores-Saavedra J*: Stromal tumor of the gall-bladder with phenotype of interstitial cells of Cajal: a previously unrecognized neoplasm. *Am J Surg Pathol* 2000; 24: 1420-23.
14. *Hinescu ME, Ardeleanu C, Gherghiceanu M* et al.: Interstitial Cajal-like cells in human gall-bladder. *J Mol Histol* 2007; 38: 275-84.
15. *Ahmadi O, de L Nicholson M, Gould M*, et al.: Interstitial cells of Cajal are present in human extrahepatic bile ducts. *J Gastroenterol Hepatol* 2010; 25(2): 277-85.
16. *Streutker CJ, Huizinga JD, Driman DK* et al.: Interstitial cells of Cajal in health and disease. Part I: Normal ICC structure and function with associate motility disorders. *Histopathology* 2007; 50: 176-89.
17. *Negreanu LM, Assor P, Mateescu B* et al.: Interstitial cells of Cajal in the gut – a gastroenterologist's point of view. *World J Gastroenterol* 2008; 14(41): 6285-88.
18. *Forster J, Damjanov I, Lin Z* et al.: Absence of the interstitial cells of Cajal in patients with gastroparesis and correlation with clinical findings. *J Gastrointest Surg* 2005; 9(1): 102-08.
19. *Festi D, Frabboni R, Bazzoli F* et al.: Gallbladder motility in cholesterol gallstone disease. Effect of ursodeoxycholic acid administration and gallstone dissolution. *Gastroenterology* 1990; 98: 1779-85.
20. *Portincasa P, Di Ciaula A, Vendemiale G* et al.: Gall-bladder motility and cholesterol crystallization in bile from patients with pigment and cholesterol gallstones. *Eur J of Clinical Investigation* 2000; 30: 317-24.
21. *Pomeranz IS, Shaffer EA*: Abnormal gall-bladder emptying in a subgroup of patients with gallstones. *Gastroenterol* 1985; 88: 787-91.
22. *Pasternak A, Gil K, Matyja A* et al.: Loss of gall-bladder interstitial Cajal-like cells in patients with cholelithiasis. *Neurogastroenterol Motil* 2013; 25(1): e17-e24.
23. *Juan Rodés* et al.: Textbook of Hepatology. 3rd ed. *Blackwell Publishing* 2007.
24. *Wang DQ, Schmitz F, Kopin AS* et al.: Targeted disruption of the murine cholecystokinin-1 receptor promotes intestinal cholesterol absorption and susceptibility to cholesterol cholelithiasis. *J Clin Invest* 2004; 114: 521-28.
25. *Hu WM, Luo HS, Ding XW:* Expression of *C-kit* messenger ribonucleic acid and *C-kit* protein in the gallbladders in guinea pigs of high cholesterol diet. *Dig Dis Sci* 2009; 54(8): 1651-55.
26. *Admirand WH, Small DM*: The physicochemical basis of cholesterol gall-stone formation in man. *J Clin Invest* 1968; 47: 1045-52.
27. *Vlahcevic ZR, Bell CC, Swell L*: Significance of the liver in the production of the lithogenic bile in man. *Gastroenterology* 1970; 59: 62-69.
28. *Shoda J, He BF, Tanaka N* et al.: Increase of deoxycholate in supersaturated bile of patients with cholesterol gall-stone disease and its correlation with de novo syntheses of cholesterol and bile acids in liver, gall-bladder emptying, and small intestine transit. *Hepatology* 1995; 21: 1291-1302.
29. *Jimi M, Nagamitsu S*: Lithogenic bile. *Gastroenterol Japonica* 1976; 11(2): 111-15.
30. *Carey ML, Small DM*: The physical chemistry of cholesterol solubility in bile: Relationship to gallstone formation and dissolution in man. *J Clin Invest* 1978; 61: 998-1026.
31. *Holan KR, Holzbach RJ, Hermann RE:* Nucleation time: a key factor in the pathogenesis of cholesterol gall-stone disease. *Gastroenterology* 1979; 77: 611-17.

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