

Highly selective behaviour of colon adenoma after administration of EMR composition and its HCT116-based model.

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Abstract

When applying the improved composition of the solution used during endoscopic mucosal resection (EMR), we observed unexpectedly large and quantitatively significant differences in adenoma response vs. healthy tissue of the surrounding GIT tract, namely, the selective reaction enhancing the adenoma volume and differentiated colour. The *in vitro* experiments on the model neoplasia cell line HCT116 suggest that the robust differences in the response of starving cells can be traced down principally to tetrastarch digestion and the enhanced metabolic rate of neoplastic cells. The neoplastic tissue grows into several intestine layers so that submucosal injection of iso-oncotic tetrastarch compound leads to degradation of starch and production of oncotic molecules in submucosa transported by facilitated transport into the neoplastic tissue. The colour distinction is due to concentration differences of the reporting dye between three separated compartments, further enhancing the utility of the contrasting mixture. The diffusion dynamics shall be tuneable by optimizing starch composition, improving desirable pharmacokinetics.

Introduction

Neoplasms of the gastrointestinal tract are tumour-transformed tissues that can gradually progress into malignant tumours. The therapy of such neoplasms is usually radical and involves surgical procedure incision of detected neoplasms. The position, shape, and size of the neoplasm are typically corroborated using the endoscopic method. The endoscopist uses an endoscopic optical probe to localize the position of such polyps along the gastrointestinal tract.

The neoplasia that is not embedded into the deeper layers of the tissue can be removed during the same endoscopic examination session by using endoscopic mucosal resection (EMR). By resection through the middle or deeper part of the submucosal layer, EMR allows complete and curative resection of the diseased mucosa. For the indicated early stages of progression, EMR can be accomplished with minimal cost, morbidity, and mortality and can improve the long-term quality of life of patients^{1,2}.

Injection of a suitable solution is used to separate the neoplasm from the muscularis propria. If the lesion is distinct visually, it usually means that there is no deep submucosal invasion. On the other hand, the “non-lifting sign” has been found to have 100% sensitivity, 99% specificity, and 83% positive predictive value for invasive carcinoma³.

Successful elevation of neoplasms allows the application of polypectomic loop and control of the incision process. The composition of the injection solution for EMR use is not standardized⁴, but efforts have been made to improve the coagulant and colouring properties of such compositions. In the literature, using a highly concentrated salt solution as a coagulant with the addition of adrenalin or epinephrine has been reported⁵. Alternatively, isotonic saline solution with the addition of derivatives of cellulose, succinyl gelatine, glycerol, and fibrinogen has been formulated to slow diffusion⁴. The

physiological solution with the addition of methylene blue or sodium salt of indigotindisulfonate was applied as a visual tool to stain the neoplasm⁵.

The disadvantage of currently utilised solutions for diagnostics and surgical treatment of neoplasms of the GI tract is mainly the short time of the elevation of the lesion (separation of adenoma) and only moderate visual distinction from surrounding tissue. The lifetime of such raised adenoma is determined by fast diffusion of injected solution, which leads to the disappearance of adenoma without its colour distinction. When an auxiliary colouring agent is used, the colour boundary between the adenoma and healthy tissue is dispersed due to the rapid diffusion of the colouring agent into both volumes.

During our search for the optimal solution composition, we found a very pronounced effect when a specific injection solution - composed of components approved for systemic administration and administered under valid law - was applied as a submucosal injection into the neighbourhood of suspected neoplastic tissue as a part of the EMR procedure. Several polyps were raised above the injected tissue, lasting several minutes and forming pronounced stems, allowing for comfortable application of polypectomic loop. The colouring compound formed three differently coloured volumes - injected tissue, neoplastic tissue, and the thin boundary between them, further helping to diagnose the extent of neoplastic tissue and its level of embeddedness. The new empirically found composition clearly and repeatedly improved the EMR procedure compared with the actual clinical practice at the hospital and compared with the literature.

In our attempt to elucidate the behaviour observed during EMR, we performed *in vitro* experiments on the HCT116 (human colorectal carcinoma) cell line subjected to the conditions present during endoscopy. In this paper, we report our observations and suggest the mechanism of action based on our limited experiments and known facts.

Materials And Methods

Preparation of EMR solution. The EMR solution we used consists of three components: the physiological (saline) solution, in which the visual contrasting aid - the sodium salt of [4-(alpha-(4-diethylaminophenyl)-5-hydroxy-2,4disulfophenyl-methylidene)-2,5cyclohexadiene-1-ylidene]diethylammonium hydroxide inner salt (PATENTEBLAU V sol. inj. 2ml/50mg, GUERBET, France)⁶ and the colloid modulator of velocity - a Hydroxyethyl starch (HES) (VOLUVEN® sol. inf. Then, 1x500 ml, Fresenius Kabi, Bad Homburg, Germany)⁷ was dissolved. We prepared a mixture of EMR composition by mixing 500 ml of isotonic saline solution with 1 ml of colour constituent (PATENTEBLAU V) followed by diluting with a HES drawn up into the syringe with Combi-Stopper (syringe bung) in the ratio 3:7 under aseptic conditions and apyrogenic (dilution closed path) in a laminar flow hood.

The selection of suitable patients and administration protocol. From March 1st to June 30th, 2014, 62 patients (19 females and 43 males of average age 56,8 and 61,1 years, respectively) were indicated for EMR at the Department of Gastroenterology of the Central Military Hospital.

The patients were selected for EMR either directly at the Gastroenterology Department or following doctor referral at another department of gastroenterology in Slovakia due to a preventive examination. The patients were diagnosed during routine colonoscopy with sessile or semisessile adenoma of the colon and rectum (diagnosis group ICD-10 codes D 12.0, D12.2-D12.8). All 62 patients undergoing polypectomy at The Department of Gastroenterology of the Central Military Hospital during this period were administered the new EMR solution. Exclusion criteria (EMR contraindication) were thrombocytes (PLT) less than $50 \times 10^9/l$, prothrombin time ratio (INR) more than 1.4, discontinuation of anticoagulant or dual antiaggregant therapy less than seven days before EMR. The number of patients (sample size $n = 62$) who underwent polypectomy with EMR intervention was typical for a 4-month period at the hospital. There were no other selection and/or exclusion criteria applied. In other words, all patients within the period were administered the new EMR composition, with probability $p = 1.0$.

Informed consent

was obtained from all patients. The procedure was approved by the Ethical Committee of the Central Military Hospital SNP Ružomberok, Slovakia, according to valid Slovak law.

The administration protocol followed the routinely used procedure and was performed in accordance with the relevant guidelines and regulations. The only change in the standard application protocol was replacing the EMR composition consisting of a physiological (saline) solution, PATENTEBLAU V, and adrenaline with the new composition.

During the endoscopic session, the close neighbourhood of suspected tissues was injected by submucosal injection via the endoscopic channel in a 1–20 ml volume (depending on neoplasm size), leading to bolus and subsequent volume and colour changes. The elevated polyps were removed using a polypectomic loop, and removed tissue was analysed for histology. As a rule, several polyps became visible with the formed stem lasting several minutes and were removed in a single step during the same endoscopic session.

Cell line model. The human cancer cell line HCT116 (large intestine) was purchased from American Type Culture Collection (ATCC, CCL-247™) and cultured in RPMI 1640 growth medium (Biosera, Kansas City, MO, United States). The growth medium was supplemented with 10% foetal bovine serum (FBS) and 1x HyClone™ Antibiotic/Antimycotic solution (GE Healthcare, Little Chalfont, UK) and maintained in an atmosphere containing 5% CO₂ in humidified air at 37°C. Before experiments, the viability of cells was analyzed by trypan blue assay. The cell line was authenticated by the ATCC Laboratory Authentication Service using Sanger sequencing. ATCC declared no Mycoplasma contamination. Before experiments, cell specimens were tested again for Mycoplasma contamination by DNA staining and fluorescence microscopy visualization with negative results.

For experiments, cells were seeded in 96-well low-density plates and maintained in complete culture medium for 24 hours. The cells were then starved in saline solution without nutrients for 24 hours, mimicking the patient preparation before EMR surgery.

The initial cell culture was grown to 10 thousand cells per well, forming plaques. During starvation, some of the cells detached and floated freely in the medium. We removed free-floating cells with the part of liquid media, and the removed volume was then restored by adding the same volume of saline solution. Even though the saline solution detaches part of the starving cells, a significant fraction of the starving cells remain attached to the density plate after adding the saline solution.

Adding the contrasting composition. The EMR composition was applied for two groups of cells – nonstarving and after 24 hours starving in saline solution. After 24 hours, the nonstarving cells adhered to the density plate, while in starving cells, the free-floating cells had to be removed, and the media was replenished by saline solution first. In both cases, the last step of the procedure consisted of replacing the saline medium with an EMR composition. For starving cells, approximately half of the volume of saline was replaced by EMR. Subsequently, we prepared a set of substances without a colour constituent. In all cases, the cells exposed to the solution were followed by 10 minutes of live video flow on a Cytation 3 Cell Imaging multimode sensor (BioTek Instruments, Inc.) and evaluated visually for cell count, cell volume, and cell shape change.

Results

EMR use. Under the conditions specified above, the EMR composition was applied to 62 patients with adenoma qualifying for EMR. The typical reaction to the administration of contrasting composition by submucosal injection of adenoma larger than approximately 20 mm is depicted in Figs. 1–4. EMR composition provides colour-contrasting differences between the tissues. The normal tissue is light blue. The thin boundary of dark blue colour is formed between the adenoma and the healthy tissue, while the adenoma is not coloured. By injecting this EMR composition into the submucosal layer, adenomatous polyps noticeably increase their volume and elevate above the surface for 10–25 minutes, prolonging the time window for resection. At the same time, sharp colour differences between the healthy and neoplastic tissues and the boundary between them can be observed. Colour distinction and the increased volume of the elevated polyp thus improve the precision and quality of polypectomic surgery. The observed reaction of adenomatous polyps to new EMR composition differs from reaction to commonly used solutions where the volume changes are in the submucosa and the colour contrast – if colouring component is used – is more diffuse and not so sharply pronounced.

In vitro model. For nonstarving cells, we observed no significant changes when the saline solution was replaced by EMR solution. This is in sharp contrast with the reaction of starving HCT116 cells. In repeated experiments, HCT116 cells starved for 24 hours in saline solution detached entirely from the density plate. Figure 5 and Fig. 6 show rare cases where the group of detached cells remained partially attached so that the cells could still be localized and recognized. In other cases, the reaction of cells was so pronounced that the cells completely disappeared from the visual field of the microscope and were not identified. For the cells in both Fig. 5 and Fig. 6, we estimated cell volume changes of approximately 4%. Nevertheless, for most experiments, the expected volume expansion leading to the separation of cells must be higher but was not quantified.

The experiments repeated after two weeks with different HCT116 cultures confirmed the same results.

Discussion

Our original attempt to improve the EMR composition led us to observe significant differences in the response exclusively for starving cells. Starvation of *in vitro* cell culture was achieved by leaving the cells 24 hours in saline only (i.e., no standard fasting medium). In clinical practice, starvation of the cells is accomplished by the patient abstaining from oral food and fluid intake for 24 hours (*nill per os*) before EMR.

The only component of EMR composition capable of triggering such a reaction is HES. The colon and rectum are not involved in starch digestion, so the differences must be due to the starch processing capabilities of neoplastic tissue.

The administration of EMR composition into submucosa exposes the embedded part of the adenomatous polyp to HES-chemically modified starch. HES, as a large macromolecule (average molecular weight 130 kDa)⁹, is expected to diffuse slowly in the submucosa. Indeed, this can be seen by comparison of Fig. 2 and Fig. 3 separated in time by 150 seconds.

We expect to find alpha-amylases (or functionally equivalent enzymes) present in the submucosa and released to the environment by starving neoplastic cells. While we found no direct data supporting the expression of starch-processing alpha-amylases in colon adenomas, they are reported in similar and thus related lung adenocarcinomas¹⁰. Under the conditions indicated for EMR, adenoma polyps of size approximately 20 mm are still mostly benign – less than 10% further progress along a known adenoma-carcinoma sequence¹¹. The probability of 62 EMR resections being all carcinoma is thus very low, and we shall assume that adenomas already express alpha-amylases in sufficient amounts. Due to the applied protocol, we can assume that alpha-amylases are present even when part of the liquid (the saline solution used for starving) is removed; on the time scale of experiments, the *de novo* synthesis of alpha-amylases should not manifest within tens of seconds of HCT116 exposure to EMR.

If alpha-amylases (or their functional equivalents) are present in the submucosa, the initial volume of iso-osmotic HES can be degraded, and progressively smaller hydrolysis fragments form in the submucosa. In contrast to blood plasma, even gradual hydrolysis to fragments below the renal threshold (45–60 kDa)¹² remains active in the submucosa. As a result, a continuous supply of glucose and hydroxyethyl glucose (hydroxyethylated at C2/C6 ratio 9.05:1)⁹ is delivered into the submucosa. The dynamic mixture of fragments, including the final monosaccharide product of alpha-amylases, is produced in the submucosa, contributing to the rise of oncotic pressure.

Without the transport of monosaccharides, oncotic pressure manifests as a volume increase in the submucosa. In our observation, however, the dominant volume changes were observed in the volume of neoplastic tissue. A similar volume change was also observable *in vitro* in adherent 2D plaques formed

by the model HCT116 line. While carcinomas are known for enhanced expression of glucose transporters, adenomas must gradually acquire the capability in the early stage of adenoma-carcinoma transition. It seems thus reasonable that polyps of approximately 20 mm size, indicated for EMR, already possess an enhanced amount of glucose transporters, particularly GLUT1^{13,14}.

In healthy tissue, the transport of monosaccharides proceeds in the direction from the lumen to the serosa, and the transport for an excessive concentration of monosaccharides is facilitated. The adenomas expose the same surface present to the lumen to the submucosa so that the direction of facilitated transport of monosaccharides is reverted.

The facilitated transport of sugars into cells is specific because different saccharides are transported with different efficiencies. Additionally, hydroxyethyl glucoses are expected to be transported into cells less efficiently than anhydrous glucose. Thus, the depletion of the pool of oncotic pressure generating saccharides in the submucosa volume follows complex kinetics, well beyond our current focus.

Nevertheless, the *in vitro* HCT116 model cells eagerly transport oncotic molecules, as demonstrated by violent volume changes leading to loss of cell adherence. This kinetics can be why the polyp rather than the submucosa increases the volume in clinical observation/application.

EMR administration details. The EMR protocol that was applied corresponds to the submucosal injection of the EMR composition, where the actual composition is delivered into the submucosa.

The resection starts by administration of the EMR composition below the adenoma into the healthy submucosa. The preparation of the patient included 24 hours of fasting.

The HCT116 cell line forming 2D cell plaques is often utilized as an imperfect model of human colon cancer cells. Adenomatous polyps larger than 20 mm have an increased probability of being malignant¹⁵. Thus, HCT116 cells should be reasonably representative of the most numerous and voluminous cell composition of the expected stages of adenoma progression during reported EMR.

Conclusion

On the practical side, the modification of EMR composition and the application protocol lower the application barrier for EMR by improving the comfort and precision of the EMR. By clearly delineating the polyp boundary and the volume changes, lasting a longer time, EMR can be performed with less time-related stress and a lower risk of unwanted complications.

Moreover, our work indicates – somewhat surprisingly – that adenomas in the early stage of transition adenoma-carcinoma already express alpha-amylases and exhibit elevated glucose transport responsible for volume changes. This provides the opportunity for functional diagnostics similar in spirit to fluorodeoxyglucose contrasting in PET².

The explanation we present suggests modifying the application protocol by taking advantage of different pharmacokinetics based on controlled and tuneable development of oncotic pressure. This can probably be used to develop more selective drug delivery to more specifically characterised target tissues.

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Declarations

Conflict of interest

We declare that none of the authors have competing financial or nonfinancial interests as defined by Nature Portfolio.

Data availability

The datasets generated during the current study are not publicly available because they contain sensitive patients data. Archived hospital records, including patients age, sex, ATC diagnoses and other relevant pre-colonoscopy records, histology of the extracted tissues taken during the EMR as well as the number of repeated colonoscopy sessions are available after anonymization from authors at reasonable request.

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Figures

Figure 1

Endoscopic findings before submucosal injection of the EMR composition. The size of the polyp is about 4.5 cm. (Time T+0 seconds immediately before submucosal injection)

Figure 2

Submucosal injection of EMR composition below the adenoma. The surrounding healthy tissue is light blue in color, and the adenoma color is nearly unchanged. Note the rise of volume of the adenoma. (Time T+150 seconds after submucosal injection of EMR composition)

Figure 3

The thin dark blue boundary is formed between the adenoma and surrounding healthy tissue. Polypectomic loop is inserted. Note the visible progress of diffusion of the colour in the healthy tissue. (Time T+300 seconds after submucosal injection of EMR composition)

Figure 4

Resection and complete removal of the GI adenoma. The mucosa and light blue submucosa below are visible after resection. (Time+ 330 seconds after submucosal injection of EMR composition)

Figure 5

Group of starving HCT116 cells with free-floating cells removed (bright-field image)

Figure 6

The same field of view as in Fig. 5 after the cells were exposed to combination saline solution and HES at a ratio of 3:7. After T+30 seconds, the cells detach and, for most part, float away from the field of view (bright field image)