Subclinical myopathy in early stage colorectal cancer at disease onset: No evidence of inflammatory cells infiltration in the skeletal muscle biopsies harvested during diagnostic laparoscopy

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Abstract

Skeletal muscle biopsies harvested from asymptomatic patients affected with early stage colorectal cancer present centralized and central myonuclei, a feature that may be indicative of tumor associated muscle disorders, potentially leading to cachexia. Biopsy of the rectus abdominis muscle were harvested during elective laparoscopic tumor resection, before any chemotherapeutic treatment, thus excluding the most trivial etiologic hypothesis. On the other hands, anesthetics and other medications could induce a mild myopathy that we may exclude on the basis of morphometric analyses, ATPase histochimistry, and immunohistochemical studies using antibodies directed to N-CAM and to MHC-emb, two sound makers of muscle denervation and damage-induced muscle regeneration. Here we present immunohistochemical evidence of no infiltration of the muscle tissue with inflammatory cells, thus excluding also the trivial explanation of a trauma-induced myopathy during laparoscopy. Factors and mechanisms of this early cancer-associated mild myopathy are yet unknown. Follow-up of patients will grant the clinical relevance of these surprising observations and their predictive value of an incoming severe muscle wasting syndrome.

Key Words: anti-CD45; cachexia; centronucleated myopathy; colorectal cancer; myosistis; muscle; rectus abdominis

Skeletal muscle from patients affected with cancer may early or later undergo morphological changes either due to the treatments of the tumor, such as radiation or chemotherapy, unrecognized factors and mechanisms, including immune-inflammatory factors of tumor origin [1,10]. Morphological changes are mainly associated to muscle atrophy as a consequence of the imbalance between the rate of protein synthesis and degradation. It has been shown that in cachetic tumor-bearing mice, the inflammatory microenvironment of the tumor, which can spread systemically, can also initiate muscle necrosis and degeneration, followed by non-compensatory regeneration, characterized by the infiltration of inflammatory cells, centralized nuclei and the expression of markers of muscle regeneration, such as the embryonic isoform of myosin heavy chain (MHC-emb) [17]. Beside the late event of severe muscle wasting, some tumor could be associated with development of earlier myopathies as part of paraneoplastic syndromes. It has been shown that idiopathic inflammatory myopathies are associated to a variety of solid tumors.
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(breast, lung, gastrointestinal, colorectal). In the so called “paraneoplastic” inflammatory myopathies, the surgical removal or pharmacological treatment of the cancer results in the disappearance of clinical symptoms of the disease, underling the tight association between cancer and the development or progression of this type of myositis [20]. However, the incidence and the onset timing of the inflammatory myopathies that are the expression of an occult or diagnosed cancer is not defined yet [2].

We previously described the surprisingly observation that skeletal muscle biopsies harvested during diagnostic laparoscopy from asymptomatic patients affected with early stage colorectal cancer present centralized and central myonuclei [24]. Our previous observation that internal nuclei are preferentially identified in fast myofibers [24], may support the hypothesis that this is not an accidental phenomenon related to the anaesthetics, rather it is an undefined mechanism (i.e. the tumor load and/or the tumor-associated microenvironmet), to which myofibers sub-types possibly respond. In these muscle biopsies of some regenerating the presence of MHCUemb and NUCAM positive myofibers is also strong evidence that the myopathy is not related to the intraoperative modality of biopsy’s harvesting [24].

The main objective of the present report was to confirm in the skeletal muscle of patients affected with early stage colorectal cancer (before any chemotherapy treatment and without other associated disorders) that the detected myopathic features are not related to the trauma of the laparoscopic harvesting of the rectus abdominis biopsy. Thus, we performed immunohistochemical analyses of the presence of infiltrating inflammatory cell (positive after anti-CD45 immunostaining) in those muscle biopsies presenting a high percentage of centralized and central myonuclei.

Materials and Methods

Subjects characteristics

Patients with an early diagnosis of colorectal cancer (n=10) were enrolled for this study. Main demographic and clinical data are reported in Table 1 in [24]. All patients underwent elective laparoscopic colorectal resection. None of the patients underwent chemotherapy before resection, or was under treatment with drugs known to induce myopathy as main side effect. Healthy subjects (n=10) were enrolled as study control group. All enrolled subjects were volunteers that signed an informed consent. The mean age of enrolled patients was 63.3±10.9 (years±SD). The mean disease duration at diagnosis relative to the onset of first clinical symptoms was 5.5±10.7 months (Table 1 in [24]). Time elapsed between diagnosis and the surgical excision of the tumor as well as of the muscle biopsy, was in all patients less than one week. The mean age of control subjects was 22.7±2.6 (years±SD). All subjects were asymptomatic for muscle pain, fatigue, and weakness.

Muscle biopsies

All patients underwent open biopsy of the rectus abdominis muscle, during elective laparoscopic colorectal resection. Needle muscle biopsies, harvested according to Kern et al. [15] from the vastus lateralis of healthy subjects were used as controls. All biopsies were immediately frozen and stored in liquid nitrogen until use.

Immunohistochemical analysis

Serial cross sections (8 µm thickness) from frozen muscle biopsies were mounted on polysine™ glass slides, air-dried and used for further analyses. For morphometric analysis the mean muscle fibers diameter was evaluated in H&E-stained cross sections in accordance to Rossini et al. [21] using Scion Image software for Windows, version Beta 4.0.2, (2000 Scion Corporation, Inc.; www.scioncorp.com).

Slides images were acquired using a Zeiss microscope connected to a Leica DC 300F camera at low magnification; identical conditions were used to acquire reference ruler images. On the acquired images, nuclei were counted and categorized as located inside or outside the muscle fiber, within the extracellular matrix. In particular, nuclei located within the muscle fiber were categorized as central when they were placed equidistant from the surrounding sarcolemma, or centralized in the case they were located more closer to the sarcolemma, but clearly separated from it by reconigsable amount of myofibrils. The number of myofibers with internally located nuclei was expressed as the percentage of the number of nuclei placed inside the fiber/number of myofibers per biopsy.

The primary murine antihuman CD45 monoclonal antibodies (murine IgG1, Dako clones 2B11 and PD7/26; Dako Corp., Carpinteria, CA), diluted to 35 mg/ml in phosphate-buffered saline with 2% bovine serum albumin (PBS/BSA), were applied to tissue sections and incubated at 4 8 C overnight in a humidified chamber; negative controls, using the murine monoclonal IgG1 MOPC-21 (Sigma Chemical Co., St. Louis, MO) diluted to 35 mg/ml, were incubated in the same fashion. The sections were incubated with the secondary antibody, a biotinylated horse antiserum IgG (Vector Laboratories, Burlingame, CA) diluted in PBS/BSA containing 5 ml/ml normal human serum, at 4-8 C for 2 h. Endogenous peroxidase activity was subsequently quenched using methanol containing 1% hydrogen peroxide. ABC standard (Vector Laboratories) was made according to the manufacturer's instructions, applied to sections, and incubated at room temperature for 1 h. Immunopositivity was localized using the chromagen diaminobenzidine (0.025% wt/vol) in PBS and 0.1% hydrogen peroxide. After washes, nuclei were counterstained for 5 min at RT with toluidine (Sigma-Aldrich, St. Louis, USA), sections were coverslipped using mounting medium (Dako, Glostrup, Denmark) and observed under a Zeiss microscope.

Results and Discussion

In our ongoing studies on muscle biopsies harvested from patients affected with autoimmune myositis, such as polymyositis and dermatomyositis [3,4,8,9,13,25], some cases of paraneoplastic forms presented myofibers with internal nuclei. We thus planned a control group of muscle biopsies harvested from patient affected with cancer at the
onset of disease. In patients’ biopsies, we observed a surprising high incidence of fibers with internally located nuclei compared to controls (see Figure 1A and B). In Fig. 1B arrows point in several instances to two internalized nuclei. Furthermore, early regenerating muscle fibers expressing MHC-emb and N-CAM were also present [23].

Fig 1. Anti-CD45 immunostain of skeletal muscle biopsies harvested from normal (A) and asymptomatic patients affected with early stage colorectal cancer (B). Arrowheads point to the inflammatory cells, that are as seldom in normal as in the patients cryosections . In panel B, internalized or central nuclei are pointed with arrows. Calibration bar=100 µm.
Interstitial (nothing to say, intramyofiber) inflammatory cells are seldom labeled by the anti-CD45 antibody, a sound marker of leukocytes. Compare Fig. 1 A, normal muscle (in which two inflammatory cells are pointed with arrowheads) to Fig. 1 B, a rectus abdominis biopsy harvested during diagnostic laparoscopy from asymptomatic patients affected with early stage colorectal cancer, in which two inflammatory cells are also pointed with arrowheads. Figure 1 B demonstrates also that a large percentage of muscle fibers with internalized nuclei have not always in proximity CD45+ inflammatory cells (arrows and arrowheads, respectively).

Surprisingly enough, in the skeletal muscle from these cancer patients we observed also that the abnormally nucleated muscle fibers are predominantly of the fast type, tough the ATPase staining of patients biopsies showed the expected slight predominance of fast-twitch fibers compared to the slow type. Fiber type shifting is not a feature of the early stage of this central-nucleated myopathy, at least at the clinical onset of the colorectal tumor [23].

During myogenesis, myotubes (the early stage of the developing multinucleated muscle fibers) have chains of central nuclei, which in normal skeletal muscle tissue migrate very soon to the periphery of the fiber, adjacent to the sarcolemma. The position of myonuclei change during fetal, neonatal and regenerative myogenesis, but the presence in adult skeletal muscles of myofibers with prominent internalized or central nuclei is described as one of the common features of muscle myogenic, neurogenic, traumatic and toxic myopathies. Internalization of the nucleus in mature fibers are encountered in a wide range of muscle diseases, such as the so called centronuclear and X-linked myotubular genetic myopathies [19], including some primary myopathies (i.e., muscle dystrophies) and autoimmune myositis [25]. The mechanism by which nuclei migrate in normal and pathologic conditions is not known, thus on the significance of internally located nuclei in pathologic muscles there are different hypotheses. As the genetic muscle diseases are concern, this alteration may represent a failure in myofiber maturation, while other hypotheses implicate neurogenic ethiology [19]. However, these are congenital myopathies or sporadic familiar diseases of adults, rarely observed in normal elderly [14].

It has been shown that in ischemic muscles some anesthetics, such as marcaine, are potent myotoxic agents, in particular for slow-type muscle fibers [18,22]. Our observation that in early colorectal cancer patients internal nuclei are preferentially identified in fast myofibers, support the hypothesis that this is not an accidental phenomenon related to laparoscopy anaesthesia, rather it is an undefined mechanism (i.e. the tumor load and/or the tumor-associated microenvironment), to which myofibers sub-types respond. The preferential distribution of nuclear internalization is also against the hypothesis of a trivial traumatic ethiology related to the surgical operation.

In addition, the detection in patients’ muscle biopsies of some regenerating MHC-emb and N-CAM positive myofibers is strong evidence that the “myopathy” is not related to the intraoperative modality of biopsy’s harvesting, but rather indicate that investigated muscle biopsies exhibit also signs of damage-induced muscle fiber regeneration that started at least days before intraoperative tumor diagnosis and resection. Indeed, the regenerating muscle fibers expressing the embryonic type of MHC result from the fusion of satellite cell-derived myoblasts, which take 14 hours to duplicate in vitro, even in the presence of myogenic growth factors [5]. Since we did not here observed any significant inflammatory cell infiltration in patients’ muscle biopsies, the presence of myofibers with internalized and central nuclei suggests that an unknown mechanism induces sub-lethal muscle damage (in particular, displacement of myonuclei) without activation of the inflammatory responses, despite the seldom events of muscle fiber death (or apoptosis?) and regeneration [23].

Altogether our findings indicate that patients affected with colorectal cancer at the clinical onset of disease display early sign of a subclinical myopathy characterized by centronucleated and regenerating myofibers. Ongoing research on follow-up of these patients will elucidate the clinical relevance of our observation. Further studies on the underlying molecular mechanisms will provide new insights on the induction of this cancer-associated myopathy and, finally, cachexia, potentially providing early biomarkers as well as specific targets for therapeutic intervention.

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