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Caractérisation et ciblage des cellules souches cancéreuses dans l'adénocarcinome gastrique

Characterization and targeting of cancer stem cells in gastric adenocarcinoma

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Titre : Caractérisation et ciblage des cellules souches cancéreuses dans l'adénocarcinome gastrique

Résumé :

Les cellules souches cancéreuses (CSC) représentent une sous-population de cellules tumorales à l'origine de l'hétérogénéité et de la croissance tumorale. Les CSC sont plus résistantes aux traitements, et à l'origine de la rechute et des métastases. L'identification des CSC constitue actuellement un enjeu majeur dans le développement de nouvelles thérapies ciblées pour inhiber la croissance tumorale et éradiquer le cancer. Dans ce travail, nous avons cherché à identifier, caractériser, et cibler les CSC dans l'adénocarcinome gastrique. Des modèles murins de xénogreffe de tumeurs primaires de patients atteints d'adénocarcinome gastrique hors cardia de types intestinal et diffus ont été développés, ainsi qu'un modèle de tumorsphere *in vitro* afin d'évaluer les capacités tumorigéniques de sous-populations tumorales. Nous avons identifié CD44 et l'aldéhyde déshydrogénase (ALDH) comme marqueurs d'enrichissement des CSC dans les 2 types d'adénocarcinomes gastriques, l'ALDH représentant un marqueur plus spécifique que CD44. Nous avons ensuite étudié l'effet de l'acide rétinoïque tout trans (ATRA), et nous avons montré que l'ATRA inhibe la formation et la croissance des tumorspheres *in vitro* ainsi que la croissance tumorale *in vivo*. Cet effet de l'ATRA passe par l'inhibition de l'expression des marqueurs souches et des capacités d'auto-renouvèlement des CSC. En conclusion, CD44 et ALDH sont des marqueurs de CSC dans les adénocarcinomes gastriques hors cardia de types intestinal et diffus, et le traitement par l'ATRA constituerait une stratégie commune de traitement pour cibler spécifiquement les CSC et inhiber la croissance tumorale dans ces deux types de cancer gastrique.

Mots clés :

Cancer gastrique, cellule initiatrice de tumeur, acide rétinoïque, aldéhyde déshydrogénase, CD44, xénogreffe, tumorsphère

Title: Characterization and targeting of cancer stem cells in gastric adenocarcinoma

Abstract:

Cancer stem cells (CSCs) are a subpopulation of tumor cells at the origin of the heterogeneity and growth of tumors. CSCs are more resistant to treatment, and are responsible for relapse and metastasis. The identification of CSCs is a major challenge for the development of new targeted therapies to inhibit tumor growth and eradicate cancer. In this work, we aimed to identify, characterise, and target CSCs in gastric adenocarcinoma. Mouse models of primary tumor xenografts from intestinal and diffuse type non-cardia gastric adenocarcinomas from patients were developed, as well as an *in vitro* tumorsphere assay, to assess the tumorigenic capacity of subpopulations of tumor cells. We identified CD44 and aldehyde dehydrogenase (ALDH) as CSC enrichment markers in the two types of gastric adenocarcinoma, ALDH representing a more specific marker than CD44. We then studied the effect of All-trans retinoic acid (ATRA), and showed that it inhibited the formation and growth of tumorspheres *in vitro* and tumor growth *in vivo*. This effect of ATRA is due to the inhibition of stem marker expression and the self-renewal capacity of CSCs. In conclusion, CD44 and ALDH are effective CSC markers in intestinal and diffuse type non-cardia gastric adenocarcinomas, and treatment with ATRA provides a common treatment strategy to specifically target CSCs and inhibit tumor growth in both subtypes of this gastric cancer.

Keywords:

Gastric cancer, tumor initiating cell, retinoic acid, aldehyde dehydrogenase, CD44, xenograft, tumorsphere

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Substantial abstract (4-5 pages):

Context of the research

Gastric cancer is the fourth most common cancer in frequency and the third leading cause of cancer mortality in the world. Ninety-five percent of all gastric cancers are gastric adenocarcinomas and the main driving factor is the chronic infection by *Helicobacter pylori*.

Tumors are heterogeneous, composed of cells which are more or less differentiated and not all proliferative. The cancer stem cell (CSC) hypothesis that is now widely accepted shows that CSCs are a subpopulation of tumor cells with self-renewal and asymmetrical division properties giving rise to the more or less differentiated cells composing the tumor mass. These cells are at the origin of the heterogeneity of the tumors, and have tumor initiating properties which are responsible for tumor growth. CSCs are more resistant to treatment, and at the origin of relapse and metastasis. Several CSC markers, such as CD133, CD44 and CD24, have been characterized in tumors of different organs. More recently, detection of aldehyde dehydrogenase (ALDH) activity was also used to identify CSCs in acute myeloid leukemia (AML) and in solid tumors in the breast, lung, colon, and other organs.

In the stomach, their existence has been subject to debate. The first study performed by Takaishi *et al*. on gastric cancer cell lines proposed CD44 as a marker of gastric CSCs, but this marker was expressed in 3 out of 6 gastric cell lines, and confirmation in primary tumors was lacking. Then, the study performed by Rocco *et al*. on 12 human primary cases of gastric adenocarcinoma failed to demonstrate tumor-initiating properties of CD133+ and CD44+ sorted cells in xenograft assays in both NOD/SCID and nude immunodeficient mice.

On the other hand, the discovery of the CSCs in tumors has opened the window for the development of new anti-cancer therapies based on CSC targeting. One strategy concerns the targeting of the self-renewal and differentiation properties of CSCs. All-trans retinoic acid (ATRA) has been used in the treatment of leukemia in clinics for the past three decades for its properties to induce cell differentiation. More recently, studies suggested that ATRA induced cell differentiation via CSC targeting.

In this study, we aimed: first, to confirm the existence of CSCs and to characterize markers allowing their identification and isolation in human primary intestinal and diffuse type non-cardia gastric adenocarcinomas; and second, we assessed the effect of ATRA treatment on gastric CSC self-renewal and tumorigenic properties.

Experimental procedures

In the first part of the study, mouse xenograft models using primary non-cardia gastric adenocarcinoma from patients were successfully developed for 20% of the cases included. Among these cases, 1 diffuse and 5 intestinal histological variants showed similar histopathological features to the primary tumors after serial transplantation in mice, and were studied. The expression of 11 putative cell surface markers of CSCs described in other cancers was evaluated on these cases and on gastric cancer cell lines. Tumorigenic properties of FACS-sorted cells were evaluated by *in vitro* tumorsphere assays and *in vivo* xenografts using extreme limiting dilution assays in mice.

In the second part of the study, in order to assess the inhibitory effect of ATRA on gastric CSCs, 3 different models of ATRA treatment of gastric cancer cells were developed including 2D and 3D *in vitro* cultures and *in vivo* xenografts in NSG immunodeficient mice. ATRA-induced growth inhibition of gastric cancer cell lines in 2D *in vitro* culture was evaluated by MTT assay. A tumorsphere assay was used to assess the effect of ATRA on self-renewal in 3D *in vitro* cultures. The effect of ATRA on tumor growth was assessed on mouse xenograft models. Flow cytometry analyses were carried out to assess the effects of ATRA on cell cycle progression and apoptosis. The expression markers of cell cycle progression, apoptosis, stemness and CSCs were analyzed by RTqPCR, tumorspheres by immunofluorescence, and tumor xenografts by immunohistochemistry.

Results

Establishment of a mouse model of primary xenografts from human gastric adenocarcinomas

Fresh gastric tissue samples were collected by pathologists upon surgical resection from consenting patients who underwent gastrectomy for non-cardia gastric adenocarcinoma at the University Hospital and the Bergonié Regional Cancer Center in Bordeaux. Among the 37 tumor cases xenografted in mice, only 8 cases led to the growth of a secondary tumor; 7 were intestinal type and 1 was diffuse type according to the Lauren classification. Tumors reached a the size of 500 mm^3 16.6±3.4 weeks after the first passage (P) (P1) in mice. Among them, 6 tumor cases including 1 diffuse and 5 intestinal cases were serially transplanted successfully in mice and preserved similar histopathological features to the primary tumors of the patients until at least P5. Tumors between P2, P3 and P5, reaching a 500 mm^3 tumor size after 10±5.9, 10.6±6.9 and 7.2±0.8 weeks, were removed from the mice and freshly dissociated for each experiment in the study.

CD133 and CD44 cells with tumorigenic CSC properties identified

CD133 and CD44 expression was observed in tumor cells of both diffuse and intestinal type primary gastric adenocarcinoma. Among them, CD44 expression was restricted to a subpopulation of cells representing approximately one quarter of the tumor cells. Cell sorting based on CD133 and CD44 expression was then performed on live (7-AAD-), ESA+ (to detect human carcinoma cells) cells freshly dissociated from tumors collected from mice. Concerning the three cases studied (GC10, GC06 and GC04), both CD133+ and CD44+ FACS-sorted cells formed significantly more tumorspheres after 10 days of *in vitro* culture than their CD133- and CD44- respective counterparts. The number of tumorspheres obtained was higher in all cases with the CD44+ cells compared to the CD133+ cells. This suggested that the CD44+ cell subpopulation contained the higher number of CSCs. These FACS-sorted cells were then subcutaneously xenografted in mice in a limiting dilution assay, and tumor growth was recorded periodically. Results revealed that CD133+ cells and CD44+ cells led to the development of tumors in mice, whereas CD133- or CD44- did not or, when present, at a very lower frequency. The observed CSC frequency was between 1/105 to 1/1,911 ESA+CD133+ cells versus 1/781 to 1/66,876 ESA+CD133- cells, and between 1/29 to 1/1,020 ESA+CD44+ cells versus 1/568 to 1/28,963 ESA+CD44- cells. These results confirmed that CSCs exist in both primary diffuse and intestinal type non-cardia gastric adenocarcinomas, and they express CD133 and CD44. In addition, CD44 was more specific than CD133 for the isolation of CSCs.

ALDH is a more specific marker of gastric CSCs than CD44

The expression of 7 additional putative markers of CSCs, CD10, CD49f, CD73, CD166, CD90, CD105, which were described in carcinomas of other organs, and ALDH activity were analyzed by flow cytometry on 5 cases of primary gastric tumor xenografts and 5 gastric cancer cell lines. Results showed that ESA and CD49f were the most highly expressed markers in both cancer cell lines and primary tumors, followed by CD90 expressed in nearly half of the cells, then CD73 in more than a third of the cells. In primary tumors, CD166 was expressed in 21±13% of the tumor cells while CD105 expression and ALDH activity were detected in only 9±6% and 8±5% of the cells, respectively. CD10 was negative except in 2 of the 10 cases studied. Flow cytometry experiments of CD44 co-staining with these markers demonstrated that CD166 and CD44 were co-expressed. CD73 was expressed in a high percentage of CD44+ cells as well as in CD44- cells. CD90+ and CD105+ cells were found in equal amounts in CD44+ and CD44- cells. Interestingly, most of the ALDH+ cells expressed CD44, and the ALDH+CD44+ cells represented less than half of the CD44+ cells.

Tumorsphere assays from FACS-sorted cells showed that cells forming tumorspheres were essentially ALDH+ and CD166+, and to a lesser extent CD73+, CD90- and CD105-. Xenograft experiments in mice, in the 4 cases studied, revealed that ALDH+ cells developed tumors at a significantly higher frequency than the respective ALDH- cells (ranging between 1/38 to 1/273 for ALDH+ cells versus 1/368 to 1/21,208 ALDH- cells) and the CD133+ cells (1/105 and 1/1658, respectively) and CD44+ cells (1/49 and 1/352, respectively).

Immunohistochemistry analyses revealed that ALDH1, the main isoform of ALDH enzymes, was expressed in a smaller number of tumor cells than CD44 in most of the cases studied, except one for which its high expression did not match the low ALDH activity detected by the flow cytometry assay. *In vitro*, ALDH and CD44 were expressed in all cells composing small young tumorspheres, and in bigger and older tumorpsheres, some CD44+ALDH- cells were detected, representing more differentiated cells. Interestingly, CD44+ALDH+ cells, corresponding to CSCs, but not CD44+ALDH- cells corresponding to more differentiated cells, excluded the Hoechst 33342 stain, suggesting drug efflux properties. Verapamil treatment restored Hoechst 33342 incorporation and staining in ALDH+ cells, confirming that CD44+ALDH+ cells have drug efflux properties and may correspond to cells in the so-called side population previously proposed by others as CSCs in gastric cell lines. Finally, these results confirmed that ALDH is a more selective marker than CD133 and CD44 for the identification and isolation of CSCs in intestinal and diffuse variants of non-cardia gastric adenocarcinomas.

In the second part of this work, we assessed the effects of ATRA treatment on gastric CSCs and tumor growth of gastric primary tumors and cell lines in three complementary models including an *in vitro* mono-layer culture (2D), an *in vitro* tumorsphere assay under non-adherent culture conditions (3D), and an *in vivo* xenograft in mice.

Optimization of cell culture conditions for studying the effects of ATRA

Under 2D culture conditions of gastric cancer cell lines treated with 5 µM ATRA, MKN7, MKN74 and MKN28 responded to ATRA only under conditions of total serum deprivation, whereas others like AGS or NCI-N87 tolerated a concentration as low as 0.2% to become ATRA sensitive. Quantitative RT-PCR analyses demonstrated that RAR-γ but not RXR-α and

RXR-β were expressed at a substantial level in these cell lines, and were upregulated under serum free-conditions. With a growth inhibition of 70%, the MKN45 and MKN74 cell lines appear to be the most sensitive to ATRA under serum-free culture conditions.

Flow cytometry experiments revealed that ATRA treatment at 5 µM under serum-free conditions induced a cell cycle arrest in the G0/G1 phase.

ATRA inhibits gastric tumorsphere formation and growth

In vitro tumorsphere assays under serum-free conditions revealed that ATRA inhibited significantly the number and the size of tumorspheres. The number of tumorspheres was inversely correlated with the ATRA doses, suggesting that the drug reduced the number of CSCs with a dose-effect. Flow cytometry analyses showed that ATRA blocked cell cycle progression, in the G0/G1 phase for MKN45 and in the G2/M phase for MKN74. The downregulation of expression of A, B, E1 and D1 cyclins, CDK2, CDC25C and E2F1, which control cell cycle progression was detected by quantitative RT-PCR. In addition, an increased expression of cyclin inhibitors, P21 and P27, was observed in both cell lines as well as P16 in MKN74 cells and P53 in MKN45 cells. PCNA, an important gene which controls DNA replication in the S phase, was also downregulated in both cell lines.

This inhibitory effect of ATRA on tumorsphere formation and growth was associated with a downregulation of the expression of the CSCs marker,s CD44 and ALDH1, as well as the stemness markers, Klf4 and Sox2. These results suggest that ATRA treatment targets CSC self-renewal properties. In addition, the expression of MUC5AC, a marker of gastric differentiation, was increased; this suggests that ATRA may also favor differentiation, as reported in the treatment of promyelocytic leukemia.

ATRA inhibited the growth of gastric tumors *in vivo*

Cells from two gastric cancer cell lines (MKN45 and MKN74) and two gastric primary tumors (C06 and GC10) were subcutaneously xenografted in NSG mice, and tumor size was recorded periodically. When tumors reached the size of 100 mm^3, treatment was started and ATRA (33 or 3.3 µmol/kg) or DMSO as a control vehicule was injected once a day for 15 days. ATRA at 33 µmol/kg noticeably inhibited tumor growth, while DMSO-treated tumors continued to actively grow. The ATRA anti-tumor effect was particularly visible in the GCO6 and GC10 primary tumors, in which ATRA seemed to be effective as early as 3 days of treatment. ATRA treatment for 15 days was not sufficient to inhibit totally the growth of tumors from gastric cancer cell lines, but in some cases of primary tumors xenografts, there was no palpable residual tumor. Tumor relapse was indeed observed in all cases after stopping ATRA treatment, however it is important to note that ATRA treatment was able to maintain the tumor size up to 28 days for GC06, MKN45, and MKN74 and up to 14 days for GC06.

Immunohistochemical analysis of the residual tumors after ATRA (33 µmol/kg) or DMSO treatment showed that ATRA noticeably decreased the expression of specific gastric CSC markers including CD44 and ALDH. On other hand, the downregulation of expression of proteins involved in tumor growth including PCNA and Ki67 was also observed. ATRA-induced caspase expression was increased in three of the four cases studied.

Conclusion:

(1). CD44 and ALDH are two enrichment markers of gastric CSCs in primary tumors, and ALDH can be considered as a more specific CSC marker than CD44 in diffuse and intestinal types of non-cardia gastric adenocarcinoma.