Inhibition of hippocampal cell proliferation by methotrexate in rats is not potentiated by the presence of a tumor

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Abstract
Methotrexate is a widely used cytostatic in chemotherapy cocktails for the treatment of cancer but is associated with cognitive impairment. Previous animal studies indicated that methotrexate decreases hippocampal cell proliferation, which might contribute to the observed cognitive impairment. However, clinical studies have shown that cognitive impairment can also be noticed in some cancer patients before any systemic treatment is initiated. We aim in the present study to discern whether hippocampal cell proliferation is negatively affected by tumor growth and if the presence of a tumor amplifies the effects of methotrexate.

Buffalo rats were subcutaneously injected with PBS or Morris Hepatoma 7777 cells to induce a tumor. Two weeks after this injection the animals received an intraperitoneal injection of methotrexate or saline. Three weeks later hippocampal cell proliferation was quantified using immunohistochemical staining.

Treatment with Morris Hepatoma 7777 cells decreased the number of proliferating cells as compared to control animals. An overall tumor effect was absent mainly because methotrexate treatment significantly decreased cell proliferation with no differences between animals with or without a tumor. Neither methotrexate nor the tumor induced pica behavior.

These findings indicate that although the presence of a tumor reduces hippocampal cell proliferation it does not affect the negative effect of methotrexate on this plasticity marker. Since sickness behavior is not induced by methotrexate or tumor presence it does not play a role in the development of cognitive deficits. This study further indicates that the effects of methotrexate on brain and behavior can be studied in healthy animals.

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1. Introduction
Chemotherapy is frequently used as adjuvant treatment strategy for cancer and is associated with cognitive impairment in part of the patients [3,28]. A number of animal studies have been carried out to explore the action of cytostatics on brain and behavior, and in these studies methotrexate (MTX), a cytostatic agent used in an adjuvant chemotherapy cocktail, is often explored [6,14,18,21,22,29]. Our studies have shown that MTX has a negative effect on cognitive behavior and hippocampal cell proliferation, which was observed already shortly after treatment and lasted for several weeks [21,22]. This cell proliferation is part of the process of neurogenesis, which is thought to play an important role in learning and memory [8,12,13]. Therefore, a decrease in hippocampal cell proliferation may contribute to the cognitive impairment described above.

In animal models, the effect of cytostatics on cognitive behavior is generally examined in healthy animals, which diverges from the clinical situation. Furthermore, there are indications that a subgroup of cancer patients suffers from cognitive impairment before any systemic treatment is initiated [9,26,27]. There are currently no clear indications which neural principles might be involved in this impairment. Since we have shown in previous studies that MTX decreases hippocampal cell proliferation and impairs cogni-
tumors were removed and weighed. Brains were removed and placed in 30% sucrose solution at 4 °C. Microtome sections of the hippocampus (30 μm) were stored at -20 °C in 30% ethyleneglycol/30% glucose in PBS solution until immunohistochemical staining.

From the serial sections, every sixth section from each animal was selected and immunocytochemically stained for Ki-67 using a slightly adapted standard protocol [11]. In brief, free-floating sections were pre-treated with 0.4% H2O2 for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, CA, USA). Subsequently, sections were incubated with mouse-anti-Ki-67 (1:200, Novocastra, Newcastle upon Tyne, UK), for 4 h at 4 °C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (1:400, goat-anti-mouse, Jackson, Wet Grove, PA, USA) for 2 h at room temperature. This was followed by incubation in an avidin biotinylated peroxidase complex (1:400, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized with 0.15 mg/ml diaminobenzidine and 0.003% H2O2 solution.

After mounting the sections onto glass slides for microscopic analysis, sections were counterstained with a Mayer-haematox solution for 30 s. Counting of Ki-67 positive cells in both hemispheres of the dentate gyrus was performed under a light microscope with a magnification of 400×. Counting was performed in the subgranular layer of the dentate gyrus and counts in both blades were summed. The border of the area that was quantified was defined as the subgranular layer having a thickness of two cell-diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the 30 μm thick sections. Because every sixth section of the brain was stained, with a total of 18 slices per animal, the amount of positive cells was multiplied by 6 to get the estimated total amount of Ki-67 positive cells in the hippocampus.

3. Results

3.1. The effect of Morris Hepatoma 7777 cells and MTX on body weight gain

Body weight of all animals was measured daily and expressed as percentage of the body weight on the day of the injection with PBS or Morris Hepatoma 7777 in Fig. 1. The decrease in body weight gain in the animals treated with Morris Hepatoma 7777 indicates anorexia cachexia. After the onset of anorexia cachexia, which was also determined by a consistent reduction in food intake, the animals received MTX or saline.
Body weight of all animals after treatment with MTX or saline was measured daily and expressed as percentage of the body weight on the day of the injection. Fig. 2 shows that body weight decreased in animals treated with MTX. Animals treated with PBS/MTX started to regain body weight from day 5, whereas the tumor-bearing animals treated with MTX did not. The body weight in tumor-bearing animals treated with saline also decreased during the experiment. A main effect was found between treatment with PBS and Morris Hepatoma 7777 (F(1,24) = 4.402, P < 0.05), with the exception of days 3 and 4 which was caused by a decrease in body weight gain due to MTX treatment. A main effect was also found between treatment with saline and MTX. No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found. The * and † represent a significant difference between animals treated with PBS or Morris Hepatoma 7777, and animals treated with saline or MTX respectively.

3.2. The effect of Morris Hepatoma 7777 cells and MTX on food intake

Food intake of all animals was measured daily following MTX or saline administration and is shown in Fig. 3. The animals treated with MTX decreased food intake directly after the injection. However, in animals treated with PBS/MTX food intake reached the level of the animals treated with PBS/saline after day 6, whereas food intake of animals treated with Morris Hepatoma 7777/MTX remained at the level of the animals treated with Morris Hepatoma 7777/saline. A main effect was found between treatment with PBS and Morris Hepatoma 7777 (F(1,24) = 39.463, P < 0.001). When analyzed with an independent-sample T-test a significant difference was seen between animals treated with PBS and Morris Hepatoma 7777 from day 1 until day 9 (P < 0.05). No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

3.3. Pica behavior

Kaolin pellets were placed in the cage of the animals directly after the injection with MTX or saline to study the effect on pica behavior as a measure of sickness behavior. Carmine was added to the pellets, so the consumption of kaolin could be measured by the presence of pink faeces. The amount of pink faeces was low in all groups (average amount for all groups together 0.3 g ± 0.7 SEM), with no significant differences between the groups (data not shown).

3.4. Effect of MTX and tumor growth on hippocampal cell proliferation

Three weeks after treatment with MTX, the animals were sacrificed and the tumors were removed and weighed. There was no significant difference in the weight of the tumor between saline treated animals and animals treated with MTX. The relative tumor weight per total body weight was 1.65% ± 0.59 SEM for animals treated with saline, and 1.90% ± 0.76 SEM for animals treated with MTX. The absolute tumor weight was 5.26 ± 1.77 SEM for animals treated with saline, and 5.75 ± 2.28 SEM for animals treated with MTX.

Ki-67 positive cells in microtome sections of the hippocampus were visualized and counted (Fig. 4). The total number of Ki-67 positive cells were 1114.5 ± 304.5 SEM for animals treated with PBS/saline; 470.4 ± 106.2 SEM for animals treated with PBS/MTX; 752.4 ± 95.3 SEM for animals treated with Morris Hepatoma 7777/saline; and 440.4 ± 164.8 SEM for animals treated with Morris Hepatoma 7777/MTX. A main effect was found between treatment with saline and MTX (F(1,24) = 10.117, P < 0.01). No main effect was found between treatment with PBS and Morris Hepatoma 7777. However, this is most likely the result from the large decrease in hippocampal cell proliferation after MTX treatment which may indicate a ceiling effect. When compared individually with a one-
Fig. 4. Total number of Ki-67 positive cells in the hippocampus of animals treated with PBS/saline ( ), PBS/MTX ( ), Morris Hepatoma 7777/saline ( ), and Morris Hepatoma 7777/MTX ( ). Data are represented as mean with standard error of the mean. A main effect was found between treatment with saline and MTX (represented with *). No main effect was found between treatment with PBS and Morris Hepatoma 7777. Since this is mainly caused by the fact that MTX treatment causes a similar decrease in cell proliferation in animals with and without a tumor, we noticed that Morris Hepatoma 7777 administration did cause a significant inhibition of hippocampal cell proliferation when compared to the PBS/saline control group. No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

4. Discussion

We explored in this paper the effect of MTX on hippocampal cell proliferation in a tumor model. MTX significantly reduced the total number of Ki-67 positive cells in the hippocampus compared to control animals, indicating a decreased amount of proliferating cells. The negative effect of MTX on hippocampal cell proliferation was expected since our previous studies also showed a decrease in the total number of Ki-67 positive cells after treatment with MTX [21,22]. MTX is a dihydrofolate reductase inhibitor and has its effect on cell death by inhibiting the conversion of folic acid into tetrahydropolyfolate thereby inhibiting the synthesis of purine and thymidine [15]. In case the effects of MTX are mediated by a direct action of MTX in the brain, a sufficient amount of MTX penetrates the brain to have this effect on hippocampal cell proliferation as well.

Cognitive impairment is not only described after treatment with chemotherapy [1,3,16,20,23,28], but also in the period between diagnosis and systemic treatment [9,26,27]. The presence of a tumor in our study did appear to decrease the number of proliferating cells in the hippocampus, suggesting that this may contribute to the cognitive impairment observed in cancer patients before any treatment is initiated. In patients additional explanations for this early cognitive impairment can be found in diagnosis related emotional stress, or DNA damage and/or deficiencies in DNA repair mechanisms [9,26,27] with the latter two being linked both to the development of cancer and neurodegenerative disorders. The presence of a tumor, however, did not further enhance the negative effects of MTX on hippocampal proliferation. This finding indicates that the effects of adjuvant chemotherapy on hippocampal cell proliferation as observed in healthy animals can be extrapolated to tumor-bearing individuals.

Besides the effect of MTX and cancer on hippocampal cell proliferation, we also explored the effect on body weight gain and sickness behavior. The tumor-bearing animals in our study showed clear signs of cancer cachexia, which can be seen in the arrest or lowering of body weight gain while food intake remained stable but at a lower level compared to control animals. Anorexia cachexia is a phenomenon frequently observed in cancer patients and is associated with the early stages of the disease, serving as a diagnosis tool, as well as with the terminal stages of cancer. This side effect of cancer is described as both the loss of adipose tissue and skeletal muscle mass resulting in a high co-morbidity factor in patients. Anorexia cachexia induces metabolic changes as well, such as altered carbohydrate and protein metabolism [24]. This anorexia cachexia is, however, not related to sickness behavior since cancer cachexia did not coincide with an increase in pica behavior. Neither did MTX induce sickness behavior. MTX did reduce food intake but this was caused by diarrhea as a consequence of damage to the cells of the intestinal tract which is a side effect of MTX [7,10]. Pica behavior after MTX is also not described in the literature although other cytostatics are associated with this sickness behavior. Cisplatin is especially known to induce pica behavior [2,4,5,19,30], but also cyclophosphamide, actinomycin D, and 5-fluorouracil are associated with sickness behavior [30].

In general, we can conclude that animals treated with MTX showed a decrease in hippocampal cell proliferation, which possibly contributes to the cognitive impairment seen in some cancer patients after adjuvant chemotherapy. Since we did not find an interaction effect between MTX and cancer on hippocampal cell proliferation, our animal model in which we treat healthy animals with MTX is a validated model to test potential mechanisms that may contribute to the cognitive impairment seen after adjuvant chemotherapy treatment.

Conflicts of interest

The authors declare that they have no competing financial interests.

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References


